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THE ROLE OF CALCIUM ION IN CARDIAC
GLYCOGENOLYSIS

by



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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies, for acceptance, a thesis entitled "The Role of Calcium Ion in Cardiac Glycogenolysis", submitted by Donald Andrew Westersund in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

The hormonal control of glycogenolysis in striated muscle has been generally accepted to be of major significance in regulating the supply of endogenous substrates during periods of stress. Although it has been postulated that calcium may have a role in coupling the contractile mechanism to glycogenolysis, little evidence is available to suggest how such a process might function, or how important it may be in controlling glycogenolysis.

The experiments to be described herein were conducted on isolated, perfused rat hearts and were designed to study the possible correlation between inotropism and phosphorylase activation. The inotropic response, and changes therein, has been previously taken as an index to suggest that a mobilization of calcium ions is occurring, and that intracellular calcium ion concentrations are changing. This parameter was chosen, therefore, to study the role of calcium ion in cardiac glycogenolysis. Stimulation with paired pulses, administration of epinephrine, and alteration of the perfusing calcium ion concentration were the methods selected to produce changes in the inotropic response.

It was found that a subtle correlation between inotropism and phosphorylase activation does exist. In terms of the absolute systolic tension developed and the rapidity with which such tension is developed, it was noted that the greatest phosphorylase activation occurs when maximal (under the conditions used) systolic tension develops rapidly.

Further, it was observed that a rapid onset of maximal positive inotropism is invariably followed by a rapid offset of positive inotropism and sometimes partial contracture. These findings were interpreted to provide a model for explaining interactions between cellular calcium flux, contractile force regulation, and phosphorylase activation.

Evidence was obtained which indicates that the phosphorylase activating effect of a given, submaximal dose of epinephrine may depend as much as one-half on the increased mobilization of calcium ions which is presumably associated with the normal positive inotropic response to epinephrine. This finding suggests that the role of calcium ion in controlling striated muscle glycogenolysis may be just as important as that of the catecholamines.

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"And, as she looked around, she saw how Death, the consoler,
Laying his hand upon many a heart, had healed it for ever."

Henry Wadsworth Longfellow.

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CHAPTER I

General Introduction

A. Striated Muscle Glycogenolysis.

Striated muscle includes both cardiac and skeletal muscle forms. Both tissues respond to stress by accelerating the process of glycogenolysis. Although similar metabolic processes are involved in both skeletal and cardiac muscle, the work to be described in this thesis is concerned with cardiac muscle, and therefore particular reference is made to myocardial glycogenolysis.

The normally functioning heart uses plasma glucose⁽¹⁾ and free fatty acids⁽²⁾ as the primary substrates for its normal energetic processes. However, during stressful periods of very rapid and forceful contractions and during myocardial anoxia, ischemia, or infarction the process of glycogenolysis is rapidly accelerated to result in the liberation of glucose-1-phosphate within the cell. Glucose-1-phosphate is readily metabolized by both aerobic and anaerobic processes, and thereby provides an immediate intra-myocardial energy reserve which may be used while appropriate adjustments are being made in cardiac metabolism and/or function.^(3,4,5)

It has been generally accepted that for the most part, glycogenolysis is regulated by hormonal control mechanisms. To this extent norepinephrine⁽³⁾, glucagon^(6,7), thyroid hormones⁽⁸⁾, and epinephrine⁽⁹⁾ have been shown to induce or potentiate the process of glycogenolysis. The action of the catecholamines in causing glycogen breakdown is probably the most well understood glycogenolytic

mechanism. It has been shown that beta-adrenergic stimulation of striated muscle stimulates an adenyl cyclase system to increase the synthesis of cyclic-3',5'-adenosine monophosphate⁽⁹⁾. This cyclic-AMP then activates a protein kinase, which in turn activates phosphorylase b kinase⁽¹⁰⁾. Activated phosphorylase b kinase is responsible for the conversion of glycogen phosphorylase^a b to glycogen phosphorylase a⁽¹¹⁾. Phosphorylase is the major and first, rate-limiting enzyme acting directly to cause glycogen degradation. It has been postulated that the large number of intermediate steps between stimulation of the adenyl cyclase system by catecholamines, and activation of phosphorylase, may serve as an amplification system⁽¹²⁾.

Glycogen is a branched chain of D-glycopyranose residues; with 1,4-glucosyl unit linkages forming the chains, and 1,6-glucosyl unit linkages forming the branch points. Glycogen granules are localized within the sarcoplasmic spaces, in close proximity to both the myofibrils and the sarcoplasmic membrane system. The glycogenolytic enzymes are presumably found intracellularly near the glycogen granules.

Phosphorylase a splits glycogen 1,4-glucosyl linkages at chain terminals, releasing successive glucose-1-phosphate units to within

^a In this thesis, glycogen phosphorylase shall also be referred to simply as phosphorylase.

four glucosyl units of the branch point. Phosphorylase a, a tetramer with a molecular weight of 500,000, is considered to be the active form of the enzyme glycogen phosphorylase and expresses its activity in the presence or absence of 5'-adenosine monophosphate. On the other hand, phosphorylase b is a dimer which is active only in the presence of 5'-AMP [K_m is $3 \times 10^{-5} M^{(13)}$], and is considered to be the inactive form of the enzyme. It has been suggested that the increased activity of phosphorylase b, as induced by the presence of 5'-AMP (possible allosteric mechanism), could be of physiological significance in myocardial anoxia where tissue levels of 5'-AMP have been shown to increase^(14,15).

The degree of glycogen phosphorylase activation is expressed in terms of the percent of total phosphorylase that exists in the a form. This is done by simply determining the relative phosphorylase activities in the presence and absence of 5'-AMP.

Striated muscle phosphorylase b kinase also exists as activated and inactivated forms, the inactivated form being in excess. Inactivated phosphorylase b kinase, in the presence of calcium ion [K_m is $3 \times 10^{-6} M^{(16)}$], will exhibit maximal phosphorylase activating ability at pH 8.2 but is virtually inactive at pH 6.8. The intracellular pH of striated muscle has been determined to be in the region of 6.8 to 7.0⁽¹⁷⁾. Activated phosphorylase b kinase is active at pH 6.8, in the presence of calcium ions [K_m is $5 \times 10^{-7} M^{(16)}$]. Tissue levels of activated phosphorylase b kinase are therefore expressed

as pH-activity ratios⁽¹⁸⁾, where the activity at pH 6.8 is given as a fraction of the total activity at pH 8.2. However, this may not be a true expression of the degree of activation phosphorylase b kinase, because activated phosphorylase b kinase, in the presence of calcium ion [K_m is $2 \times 10^{-7} M$ ⁽¹⁶⁾], has been shown to be more active at pH 8.2 than at pH 6.8⁽¹⁸⁾.

K_m values for the stimulating effect of calcium ion on the phosphorylase b kinases had not been reported prior to the outset of the work to be described in this thesis. They are mentioned above for the sake of completeness.

Before discussing the history of a role for calcium ion in the coupling glycogenolysis to excitation-contraction, an attempt shall be made to briefly outline the well-documented role for calcium ion in coupling excitation with contraction.

B. Calcium Ion in Excitation-Contraction Coupling.

In cardiac muscle, depolarization of the cell membrane is accompanied by the mobilization of free calcium ions from three possible sarcomembranous sites: (1) the cell membrane, (2) the transverse tubular system, and (3) the longitudinal tubular system⁽¹⁹⁾. This mobilization results in a net flux of free calcium ions, inwardly, from extracellular or membrane-bound sites to the sarcoplasmic

spaces, where the free calcium ion concentration must exceed a threshold level of $2 \times 10^{-7} \text{ M}^{(20)}$, in order to initiate the contractile process [K_m is $1.2 \times 10^{-6} \text{ M}^{(20)}$]. The extracellular free calcium is rapidly exchangeable^(19,21) with vascular free ionized calcium. Presumably, both are in the range of about $1.36 \times 10^{-3} \text{ M}^{(22,23)}$. The extracellular free calcium ion concentration has been shown to be capable of regulating myocardial contractility.

Ionized calcium is required for the initiation, control, and maintenance of myofibril contraction. The combination of calcium ion with troponin results in the formation of a tropomyosin-calcium complex which, in the uncombined form, covers the reactive sites of meromyosin cross-bridges and actin fibrils. In other words, free calcium ion, in complexing with the secondary proteins, uncovers the reactive sites necessary for contractile interaction between the primary proteins⁽²⁰⁾. Apparently then, the force of contraction is regulated by the amount of free calcium ion released into the sarcoplasmic spaces^(19,20).

Relaxation of contracted skeletal muscle myofibrils is effected by a "relaxing factor" first recognized by Marsh in 1952⁽²⁴⁾. Ultra-centrifugation techniques were used to establish that the identity of this "relaxing factor" to be that of a sarcomembranous ATP-ase^(25,26,27). Weber et al.⁽²⁸⁾ found that these sarcomembranous "vesicles" were capable of concentrating calcium ions one hundred

thousand-fold. The above studies were later shown to be also valid for cardiac muscle^(29,30,31,32). However, controversy has arisen over the particular sarcomembranous site that this "calcium pump" is associated with. A good deal of investigation is currently being done in studying the possibility of a role for mitochondria and microsomes in muscle calcium ion metabolism⁽²⁰⁾.

It is concluded then, that two processes, namely release and uptake, regulate the sarcoplasmic free-calcium ion concentration. The typical myocardial inotropic response must therefore be a reflection of a continuous dynamic interaction between these two processes; and changes in the inotropic response may very well be related to changes in these regulatory mechanisms. This concept and its relationship to the role of calcium ion in glycogenolysis shall be discussed further in Chapter IX.

C. History of Calcium Ion in Glycogenolysis.

In 1955, Krebs and Fischer accidentally obtained the first biochemical evidence indicating that calcium ion may play a role in regulating muscle glycogenolysis. A crude skeletal muscle extract, known to contain both phosphorylase b and phosphorylase a, underwent phosphorylase activation (conversion from the b to the a form) following exposure to calcium ions that were present in an unwashed filter paper⁽¹¹⁾.

Further investigations by Krebs et al., in 1959, led them to suggest the existence of a possible role for calcium ion in the activation of phosphorylase b kinase⁽³³⁾. This work was extended by Meyer et al. in 1964⁽³⁴⁾, who suggested: (1) that a "kinase-activating factor" was necessary for the previously demonstrated calcium ion-induced activation of phosphorylase b kinase, and (2) that calcium ion appeared to be essential for the activity of phosphorylase b kinase even in the absence of the "kinase-activating factor". The significance of this second finding was apparently not realized at the time. In vitro work, done by Hammermeister et al.⁽³⁵⁾ in 1965, and Drummond and Duncan⁽³⁶⁾ in 1968, supported the role of the "kinase-activating factor" as proposed by Meyer et al.⁽³⁴⁾. The idea of a "kinase-activating factor" that acted in conjunction with calcium ion was subsequently widely accepted and has often been cited in the literature. However, in 1968 Huston and Krebs⁽³⁷⁾ purified the "kinase-activating factor" and demonstrated that it was in fact, a calcium ion-dependent proteolytic enzyme. This was contrary to the finding of Meyer et al. (1964)⁽³⁴⁾, who could not demonstrate proteolytic activity in the "kinase-activating factor". Because phosphorylase b kinase could also be "activated" by trypsin, Huston and Krebs concluded that proteolysis was probably the mode by which the "kinase-activating factor" and calcium ions were causing the supposed phosphorylase b kinase "activation". These workers therefore implied

that the "kinase-activating factor" probably did not have a physiologically significant role, and this view came to be supported by others in the field.

By this time however, a great deal of evidence had accumulated from studies done with in situ and isolated organ preparations, which favored the idea of a role for calcium ion in glycogenolysis. Huston and Krebs, faced with this paradox, now chose to acknowledge the significance of biochemical work done by Meyer et al. (1964)⁽³⁴⁾, which showed that calcium ion appeared to be essential for phosphorylase b kinase activity, even in the absence of the "kinase-activating factor"; and they therefore stated: "The hypothesis advanced by Meyer et al. (1964) that calcium ion may play a role in the coupling of muscle contraction to glycogenolysis through its effect on phosphorylase kinase remains very much alive."

In 1963, Haugaard⁽³⁸⁾ suggested that the activation of phosphorylase may mediate the cardiac contractile response. This suggestion was put forth by Haugaard in view of the evidence that had accumulated from investigations using in situ and isolated organ preparations^(39,40,41). Haugaard's proposal fell by the way when it was shown: (1) that low doses of epinephrine initiated positive inotropism but not phosphorylase activation^(42,43), and (2) that positive inotropism preceded phosphorylase activation^(44,45,46).

It must be emphasized that, thus far in the history of calcium ion in glycogenolysis, all evidence supporting a role for calcium ion which was separable from the role of catecholamines, stemmed

from in vitro studies supporting a conjunctive role for the "kinase-activating factor". That is to say, those investigators using isolated organ or in situ preparations in their attempts to demonstrate a role for calcium ion in glycogenolysis, had not shown that calcium ion was acting independently of adenylyl cyclase stimulation, or via the beta-adrenergic receptor. In 1967, Friesen et al.⁽⁴⁷⁾ showed that an abrupt increase in the perfusing calcium ion concentration could initiate a positive inotropic response that was followed by phosphorylase activation, in isolated rat hearts. Reserpinization, shown to deplete the cardiac catecholamine stores⁽⁴⁸⁾ did not inhibit these effects of calcium.

In 1968, immediately following the identification of the "kinase activating factor" as a proteolytic enzyme⁽³⁷⁾, Namm et al. published results of experiments which further indicated that the role of calcium ion was separable from that of epinephrine in cardiac glycogenolysis (phosphorylase activation)⁽⁴⁹⁾. Using perfused, isolated rat hearts, Namm et al. demonstrated that calcium ion was required for both the positive inotropic response to, and the phosphorylase activating effect of, epinephrine but that calcium ion was not required for the phosphorylase b kinase activating effect of epinephrine. They further showed that a three-fold increase in the calcium ion concentration of the medium could, by itself, cause modest phosphorylase activation without adenylyl cyclase activation or phosphorylase b kinase activation. They concluded: "... the mechanism of action of calcium ion is to stimulate^a the

activity of phosphorylase kinase in catalyzing the conversion of phosphorylase b to a rather than to cause activation^a of the kinase..."

Friesen et al.⁽⁵⁰⁾ (1969) published results of experiments done on isolated, perfused rat hearts which demonstrated that calcium ion mobilizing procedures produce positive inotropism followed by phosphorylase activation. The procedures involved were: (1) an abrupt three-fold increase in perfusing calcium ion levels, (2) an abrupt two-fold decrease in perfusing sodium levels, (3) paired-pulse stimulation, (4) electrically-induced positive chronotropism, and (5) administration of epinephrine. The effects of epinephrine administration were completely blocked by propranolol; but neither reserpinization nor beta-adrenergic blockade with propranolol affected the other calcium ion-mobilizing procedures. Friesen et al. were led to conclude that the data "... are consistent with the hypothesis that calcium ions have a stimulatory effect on the sequence of reactions leading to an activation of glycogen phosphorylase."⁽⁵⁰⁾

Drummond et al. (1969)⁽⁵¹⁾ compared the effects of epinephrine and electrical stimulation on cyclic AMP-levels, phosphorylase b kinase activation, and phosphorylase activation using in situ

^a The terminology of Krebs et al. is applied here: activation referring only to an effect in which a new molecular form of the enzyme is produced, and stimulation referring only to an increase in the activity of a single molecular form of the enzyme.

and isolated skeletal muscle preparations. It was found that epinephrine increased tissue cyclic-AMP levels, caused activation of phosphorylase b kinase, and also caused activation of phosphorylase. Electrical stimulation, on the other hand, caused an activation of phosphorylase, but did not increase cyclic-AMP levels nor cause phosphorylase b kinase to be activated. These findings of Drummond et al. ⁽⁵¹⁾ were not in accordance with those of Posner et al. (1965)⁽⁵²⁾, who had demonstrated electrically-induced activation of phosphorylase b kinase in skeletal muscle. This discrepancy might be explained by a slower freezing technique that was used by Posner et al. Drummond et al. were therefore led to conclude: "It is tempting to suggest that the release of calcium ion during the electrical event would not only initiate the contractile event, but act catalytically on nonactivated kinase to effect the conversion of phosphorylase b to a."

In summarizing the history of the role of calcium ion in glycogenolysis to date, a flow chart pathway of the enzymatic phosphorylase activating system is shown in Figure 1.

D. Statement of the Problem

Much of the work that has laid the foundation for a role for calcium ion in glycogenolysis was done biochemically in vitro, using somewhat purified enzyme systems. Such in vitro experimentation cannot be an end in itself; but the findings of these in vitro studies must be applicable to, and agree with, the physiological situation

found at least in the whole organ.

At the outset of the work to be described in this thesis evidence indicated that two major, related mechanisms might exist for controlling glycogenolysis in striated muscle. The first of these was the well-established hormonal control of glycogenolysis by catecholamines, and the second was a possible role for calcium ion in modulating the activity of phosphorylase b kinase. It was the object of the author's work to further elucidate the interrelationships of these two mechanism. Since it is assumed that positive inotropism is associated with an increased mobilization of calcium ions,^(19,20) a closer study of the relationships between positive inotropism and phosphorylase activation was deemed of interest. In this respect, particular interest was given to studying how much the positive inotropic response per se to epinephrine administration contributes to catecholamine-induced activation of phosphorylase. In short, physiological experiments were set up which would attempt to demonstrate the significance of a possible role for calcium ion in striated muscle glycogenolysis.

With the above objectives in mind, experiments were designed in which isolated perfused cat hearts were subjected to parameters that were intended to: (1) modify calcium ion mobilization and/or (2) effect beta-adrenergic stimulation. Complete time-course studies of the effects of the above procedures (separately and in combination),

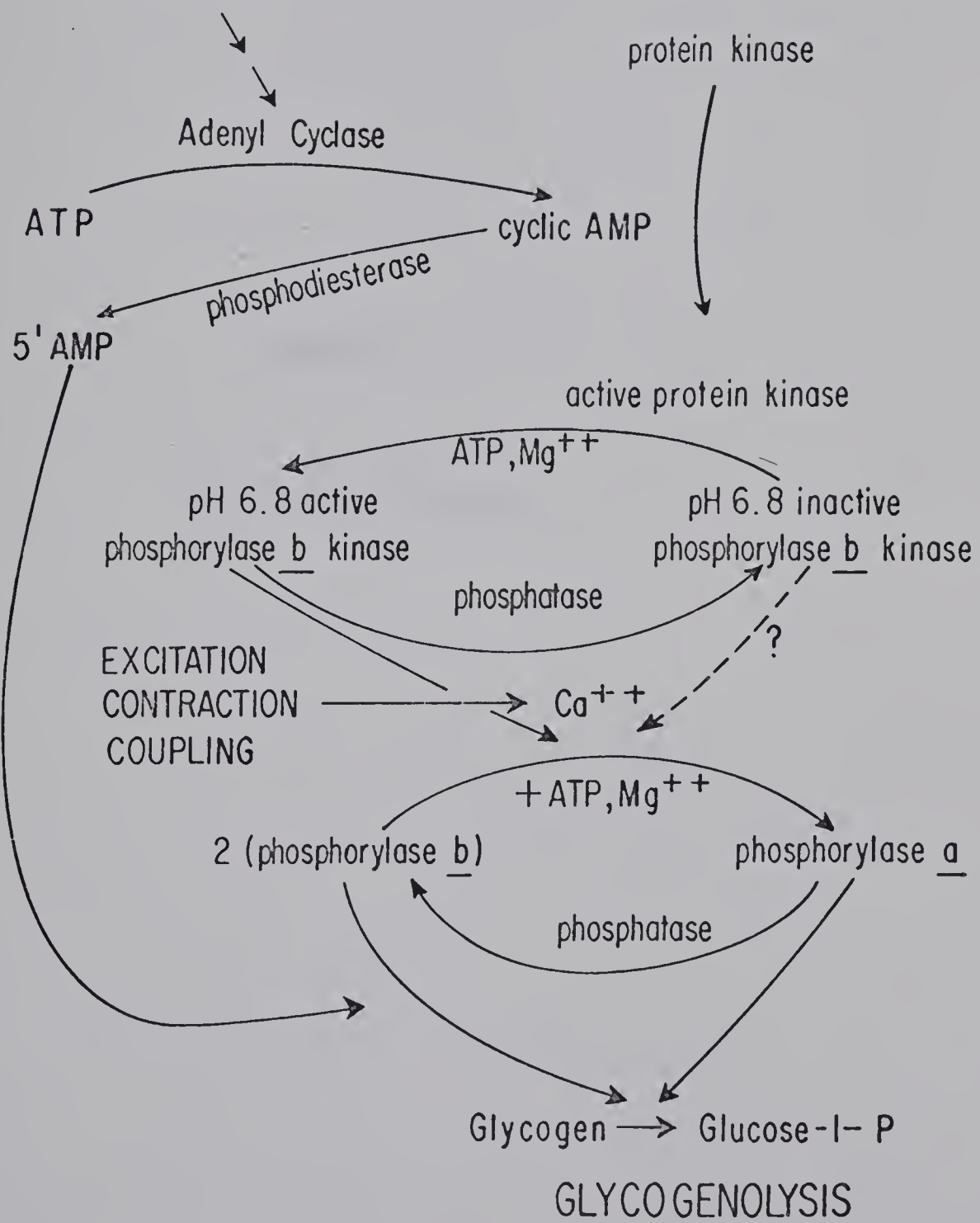
on phosphorylase activation and inotropism were undertaken in all cases.

The various procedures that were used are described in detail in Chapter II, and theoretical considerations for using such procedures are given in the Objective section of each Chapter.

Figure 1. The Glycogenolytic Pathway of Striated Muscle

Beta - adrenergic stimulation causes an adenyl cyclase system to produce cyclic-3'5'-adenosine monophosphate (cyclic-AMP) from adenosine triphosphate (ATP). Cyclic-AMP is, in turn, believed to activate a protein kinase, which is responsible for the conversion of phosphorylase b kinase from an inactivated to an activated form. Calcium ion (K_m is $5 \times 10^{-7} M$) catalytically permits activated phosphorylase b kinase to convert phosphorylase b into phosphorylase a. Both phosphorylase b, when in the presence of 5'-adenosine monophosphate (K_m for 5'AMP is $3 \times 10^{-5} M$), and phosphorylase a are capable of liberating glucose-1-phosphate moieties from glycogen.

BETA ADRENERGIC STIMULATION



CHAPTER II

Materials and Methods

A. Isolated Heart Preparation.

Female Wistar rats weighing between 150 and 200 grams were killed by a sharp blow to the head. The neck of the rat was incised from the ventral side and the spinal column severed. The thoracic cavity was opened from the sternum to the neck, and the rib cage spread apart to expose the heart. The pericardium was removed and the heart was rapidly excised, taking care to leave a sufficient length of aorta for the perfusion technique. The heart was then immediately plunged into ice-cold Tyrode's solution, and gently massaged to remove blood and prevent clotting. The time involved from the killing of the rat to immersion of the heart in cold Tyrode's solution was not more than 60 seconds.

B. Isolated Heart Perfusion.

Each heart was perfused according to the Langendorff technique (via the aorta) with Tyrode's solution (37°C) at a free-flow rate (without the heart attached) of 8 ml/min. This technique was the same as that used by Friesen et al. (47,50)

Table I lists the composition of Tyrode's solution. The constituents were purchased from J.T. Baker Chemical Co. Tyrode's solutions containing different calcium ion concentrations served where the calcium levels in the perfusion were changed. No adjustment was made for the small difference in osmolarity. Abrupt changes of the perfusions (calcium concentration) were facilitated by the

apparatus shown and described in Figure 2.

When preparing Tyrode's solutions, care was taken to aerate each solution with a gas mixture containing 95% oxygen and 5% carbon dioxide before the calcium chloride was added. This procedure reduces the pH of the solutions and prevents precipitants from forming when the calcium is added.

All solutions were aerated continuously throughout the experiment. Before each solution reached the heart, it was passed through a coarse, Pyrex sintered glass filter in order to remove insoluble impurities which could have produced blocks in the microvasculature of the heart.

C. Isolated Heart Stimulation.

1. Single Pulses

All hearts were paced electrically with single pulses during the eight minute equilibration period and also during experimental procedures where paired-pulse stimulation was not used.

In order to facilitate a normal, control rate of about 300 beats/min using electrical stimulation, it was necessary to excise the atria and stimulate only the ventricles. Platinum hook electrodes were inserted superficially into the right ventricle. When there was evidence indicating that the vasculature of the heart had been punctured by this technique the heart was discarded. Biphasic stimuli were administered at a rate of 300/min from a Grass SD5 Stimulator, using sufficient voltage (about 2.5 volts) and duration (2.0 msec) to initiate myocardial contraction.

TABLE I

Composition of Tyrode's Solution

Constituent		Amount (mmoles)
$\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$		0.36
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$		0.50
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$		varied ^a
KCl		2.50
$\text{HOCH}_2\text{CH}(\text{CHOH})_4\text{O}^{\text{b}}$		5.00
NaHCO_3		12.00
NaCl		137.0
H_2O	q.s.a.d.	1.00 liter

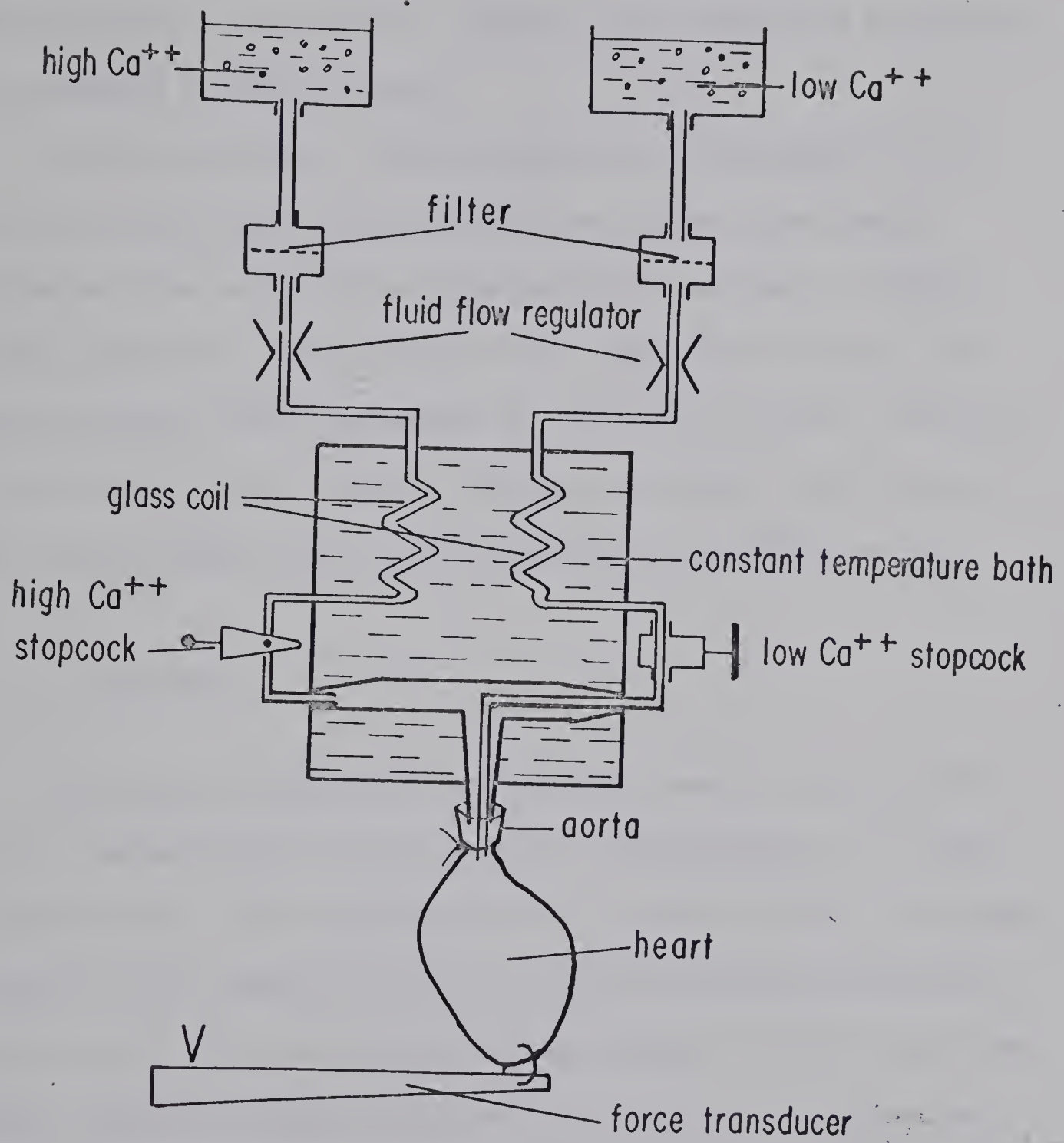
^a Calcium chloride concentration was 0.60, 0.90, 1.20, 1.80, or 2.40 mmoles.

^b D-Glucose.

Figure 2. A Simplified Diagram of the Perfusion Apparatus

The apparatus shown was used to effect an abrupt change of the perfusing Tyrode's solution from one containing a high calcium ion concentration to one containing a low calcium ion concentration.

A polyethylene tube leading directly from the low-calcium stopcock, is inserted into the aortic cannula. Thus, when the high-calcium stopcock is turned off and the low-calcium stopcock is turned on, a relatively small dead-space permits rapid infusion of the Tyrode's solution containing the low calcium ion concentration.



2. Paired Pulses

Paired pulses⁽⁵³⁾ are two electrical stimuli delivered in close time proximity to one another. Figure 3 is a simplified diagramatic representation of what is meant.

In all experiments, single pulses were administered during the eight minute equilibration period and during experimental procedures where paired-pulse stimulation was not used. However, in those experiments where paired stimuli were administered, each single pulse was simply exchanged for two paired pulses. The type of pulse used was kept constant (voltage, duration), and only the delay period between two paired pulses was varied (Chapter III).

3. Epinephrine

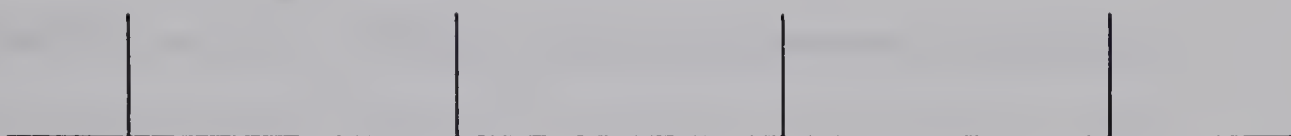
L-Epinephrine bitartrate was stored frozen in normal saline at pH 4 (buffered with acetic acid) at a concentration of 1 mg/ml of the free base. For experimental use, a stock solution containing 25 µg/ml in pH 4 normal saline was prepared extemporaneously and stored on ice. This stock solution was diluted ten-fold with iced Tyrode's solution immediately prior to injection. For injection purposes, 0.1 ml of the final solution containing 0.25 µg of epinephrine (as the free base) was used. The dose of epinephrine was slowly infused over a five-second period through a fine

Figure 3. Diagramatic Representation of Paired and Single
Pulses

"A" represents single pulses delivered at a rate of 300/min and using a potential of 2 volts.

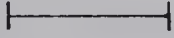
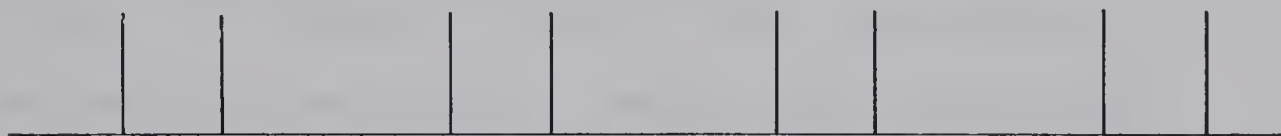
"B" represents paired pulses delivered at a rate of 300/min using a potential of 2 volts, and a delay period of 60 msec.

A



I 1 volt

B


100 msec

polyethylene tube which had been inserted into the aortic cannula.

A submaximal dose of epinephrine was employed. It is appreciated that the validity of such a dose selection may be questioned; however, it was felt that the use of a supramaximal dose may have masked the inhibitory effects of low calcium perfusion on phosphorylase activation.

D. Recording Inotropic Responses

The apex of the ventricles was sutured to a lever, which in turn was attached to a Grass FT03.C force displacement transducer. A diastolic tension of five grams was imposed on each heart. The isometric contractility was recorded on a Beckman RB Type Dynograph.

It will be noted that two methods of measuring the inotropic response were used. In Chapters III and IV, the calcium ion concentration perfusing the heart was not changed, and therefore the control (equilibrating) systolic tensions between hearts were relatively constant. In these Chapters inotropism was measured as the percent of the control systolic tension that the experimental systolic tension represented. In all other experiments, inotropism was determined as the absolute systolic tension (in grams) developed. It is suggested that the latter method better reflects, in absolute terms, the peak intracellular free calcium concentrations during systole. On the other hand, due to the biological inherent differences between individual hearts, measuring inotropism in absolute

terms rather than in relative terms involves a greater degree standard error; and therefore, in order to prove statistical significance, a greater number of samples must be obtained.

E. Phosphorylase Assay

At a predetermined time during each experiment one sample weighing between 20 and 50 mg was removed from the left ventricle of the heart using modified Wollenberger clamps prechilled in liquid nitrogen (-195°C). The clamps (containing the sample) were then immediately reimmersed in liquid nitrogen, where all tissue outside the jaws of the clamps was chipped away and discarded. The remaining sample was stored under liquid nitrogen until the time of assay.

Assay of the tissue samples for phosphorylase a and total phosphorylase (a + b) was done in duplicate as described by Cori and Illingworth ⁽⁵⁴⁾, and as modified by Mayer et al. ⁽⁵⁵⁾. Since there was no statistically significant variation of total phosphorylase levels (a + b) between control and experimental heart samples, all data are presented in terms of the percent of total phosphorylase in the a (active) form.

The glycogen to glucose-1-phosphate reaction is reversed by the presence of excess glucose-1-phosphate. Phosphorylase is capable of catalyzing the reaction in both directions. Since phosphorylase b

is only active in the presence of 5'-adenosine monophosphate [K_m is $3 \times 10^{-5} M^{(19)}$], the percent of all phosphorylase present as the a form can be measured by assaying for phosphorylase in the presence and absence of 5'-AMP. In the reversed reaction, phosphate is liberated and measured spectrophotometrically.

CHAPTER III

Varying the Delay Between Paired Pulses:
Effect on Maximal Phosphorylase Activation
and Inotropic Response

A. Introduction and Objective

Paired-pulse stimuli were used to effect positive inotropism. Electrically-induced positive inotropism has been discussed and described in detailed in the literature, and the positive inotropic effects of both paired stimulation and increased frequency stimulation have been attributed to increased intracellular free calcium ion concentrations during systole^(19,20,53,56,57). Recent studies by Suko et al. indicate that paired pulse-induced positive inotropism is comitant with increased intracellular calcium. Therefore, in studying the role of calcium ion in glycogenolysis it was felt that a correlation between positive inotropism and phosphorylase activation might exist.

As previously described, paired pulses are two pulses delivered sufficiently close together so as to produce a single contraction. The net effect of paired-pulse stimulation is the rapid production of a large, transient increase in systolic tension (positive inotropism) without a change in the heart rate.

Theoretically, the second pulse of a set of paired pulses arrives during the relative refractory period and initiates a second action potential. The effect of the second action potential is to add to the contractile effect of the first, thereby increasing the force of the single contraction (postextrasystolic potentiation). The time-delay between each of a set of paired pulses is important. If the delay period is too short, the second pulse cannot initiate a second action potential, because it arrives during the absolute refractory

period. If the delay period is too long, the second pulse arrives after the refractory period and, in initiating a second action potential, tends to produce a second, separate contraction or portion thereof.

The purpose of this series of experiments was to determine whether or not a change in the delay period between paired stimuli would change the maximal systolic tension developed with paired-pulse stimulation. If this was possible, and a graded effect on maximal positive inotropism was obtained, then it should follow that a graded effect on maximal phosphorylase activation would also be obtained.

In addition, the optimum time-delay between paired stimuli for producing maximal positive inotropism in conjunction with maximal phosphorylase activation, should also be ascertained by performing an experiment of this design.

B. Experimental Design.

Following eight minutes equilibration with Tyrode's solution containing 1.8 mM calcium, the isolated, perfused rat hearts were switched from single to paired stimuli, using delay periods of 20, 30, 40, 60, or 85 milliseconds. Preliminary investigations showed that maximal activation of phosphorylase occurs within 15 to 25 seconds after initiation of paired-pulse stimulation. Therefore, tissue sampling was done at 15, 20, and 25 seconds after switching from single to paired pulses in order to determine

the maximal phosphorylase activation with each different delay period. Measurement of the maximal positive inotropic responses were made and calculated as the percent of the control systolic tension (before paired pulses).

C. Results and Discussion.

It was found that varying the delay period between paired-pulse stimuli did not produce a graded change in the maximal positive inotropic response under these conditions⁽⁵³⁾. In fact, the positive inotropic response obtained with paired-pulse stimulation was apparently an all-or-none phenomenon in that maximal positive inotropism either did occur or it did not occur (see Figure 4).

It was found that using a short delay period of 20 msec sometimes resulted in the production of a very short-lived (atypical), positive inotropic response to paired-pulse stimulation. On the other hand, stimulation with paired pulses using a 20 msec delay period, sometimes produced no positive inotropism at all. Phosphorylase a values observed with paired-pulse stimulation using a 20 msec delay period, did not differ significantly from control values (single pulse pacing, see Figure 4). It will be appreciated that paired stimulation using a zero-time delay period, is essentially equivalent to stimulation with single pulses.

When 40, 60, and 85 msec delay periods were used, maximal positive inotropism and maximal phosphorylase activation were seen.

The values obtained using 40, 60, and 85 msec delay periods between paired stimuli however, were not statistically different (unpaired t test) from each other. That is to say, varying the delay period from 40 to 85 msec did not affect the inotropic response or phosphorylase activation that was obtained by stimulating with paired pulses. If a delay period of more than 85 msec was used, irregular second contractions appeared. The results indicated that with less than a 20 msec delay period between paired stimuli, no significant phosphorylase activation or positive inotropism was effected by stimulation with paired pulses. When the delay period was greater than 40 msec, maximal positive inotropism and phosphorylase activation (under these conditions) occurred. Repeated attempts were made to study the effect of paired pulse stimulation on these responses, using a 30 msec delay period. However, no consistent effects were seen. Stimulation with paired pulses using a 30 msec delay period, often resulted in irregular positive inotropism or ventricular fibrillation. Phosphorylase activation varied considerably. It was therefore concluded, that a "vulnerable" period exists in the region of 30 msec after the initial stimulus, where a second stimulus tends to produce unpredictable effects on the whole heart^(58,59). It is suggested that this sort of response in the whole heart may be a result of the summation of different effects in separate portions of the myocardium.

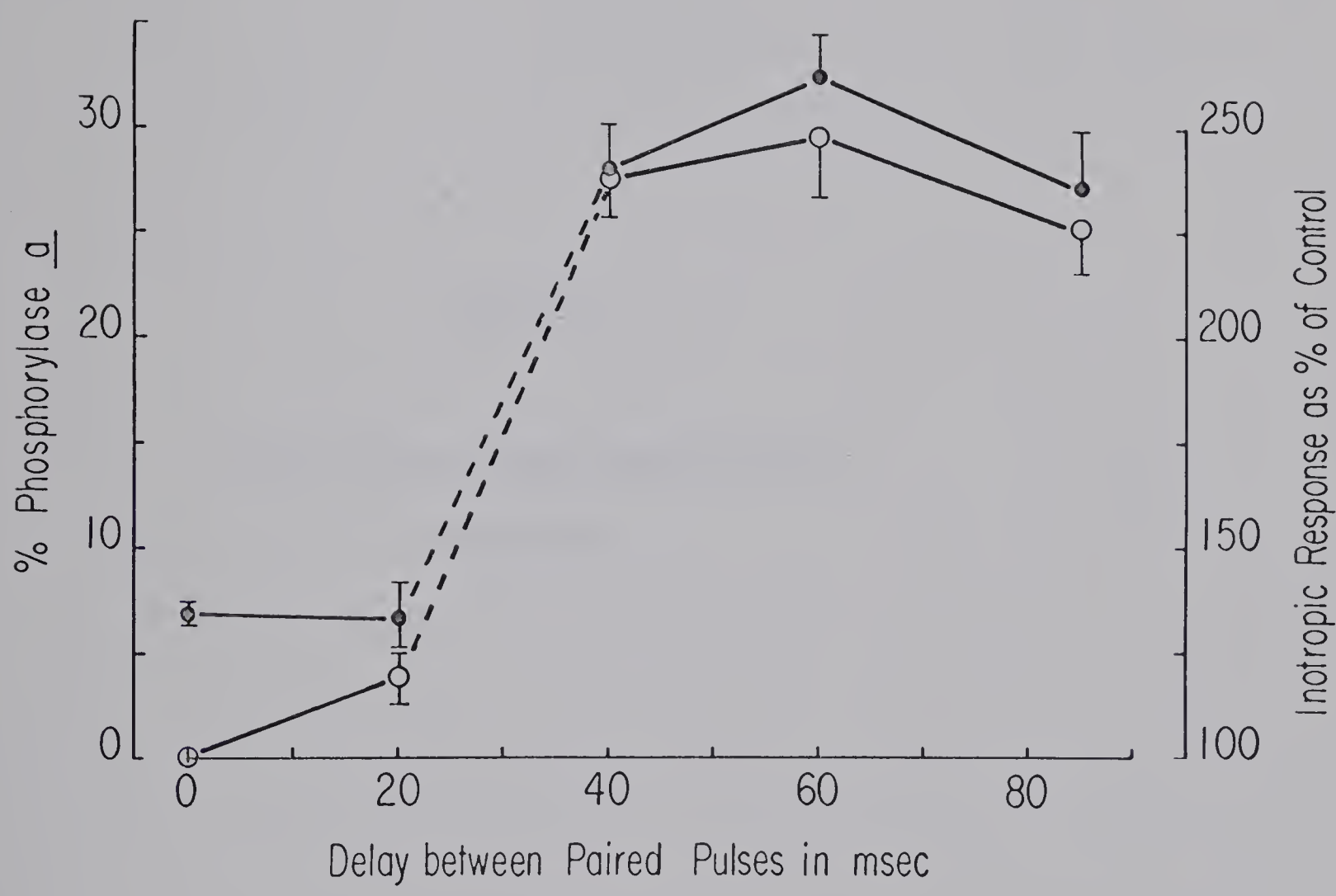
It was concluded that a graded effect on positive inotropism,

and therefore phosphorylase activation, cannot be obtained under these conditions by varying the delay period between paired-pulse stimuli. It was demonstrated that the response to paired stimuli depending on the delay period, is in fact an all-or-none phenomenon.

A delay period of 60 msec was arbitrarily chosen to be optimal for inducing positive inotropism with paired-pulse stimulation.

Figure 4. Varied Delay Between Paired-Pulse Stimuli:
Effect on Maximal Positive Inotropism and
Maximal Phosphorylase Activation

Illustrated are the maximal phosphorylase a levels (~~0~~—~~0~~) and the maximal positive inotropic responses (0—0) that were obtained following paired-pulse stimulation in which the delay period between the paired stimuli had been varied. Zero delay is synonymous with single pulse stimulation (300/min). This co-ordinate, therefore, denotes control values obtained from 32 hearts following the eight minute equilibration period (1.8 mM calcium Tyrode's). An average of seven heart perfusions were done to obtain each experimental value. I-shaped bars symbolize standard errors of the mean.



CHAPTER IV

Paired Pulse-Induced Phosphorylase

Activation

A. Continuous Paired-Pulse Stimulation.

1. Objective

The purpose of these experiments was to do a complete time-course study of the effects of paired pulse stimulation on positive inotropism in relation to phosphorylase activation.

Previous results had indicated that maximal positive inotropism was occurring before maximal phosphorylase activation. It will be recalled that an attempt to correlate positive inotropism with activation of phosphorylase is based on a role for free calcium ion in stimulating the glycogenolytic pathway. In this sense, the positive inotropic response is presumably associated with increased mobilization of free calcium ions.^(19,20) It would seem paradoxical then, that phosphorylase activation should occur after maximal positive inotropism, and at a time when the systolic tension is in fact declining. For this reason, it was deemed necessary to take a closer look at the time-course effects of the positive inotropic response to paired-pulse stimulation, in relation to phosphorylase activation.

2. Experimental Design

Following the eight minute equilibration period with 1.8 mM calcium Tyrode's solution, the isolated, perfused rat hearts were switched from single to paired stimuli (60 msec delay). Tissue samples were taken at 0, 7, 10, 15, 20, 30, and 60 seconds following

initiation of paired-pulse stimulation, and assayed for phosphorylase a levels. Measurements of the inotropic response were made directly and calculated as "percent of control" as in Chapter III.

3. Results

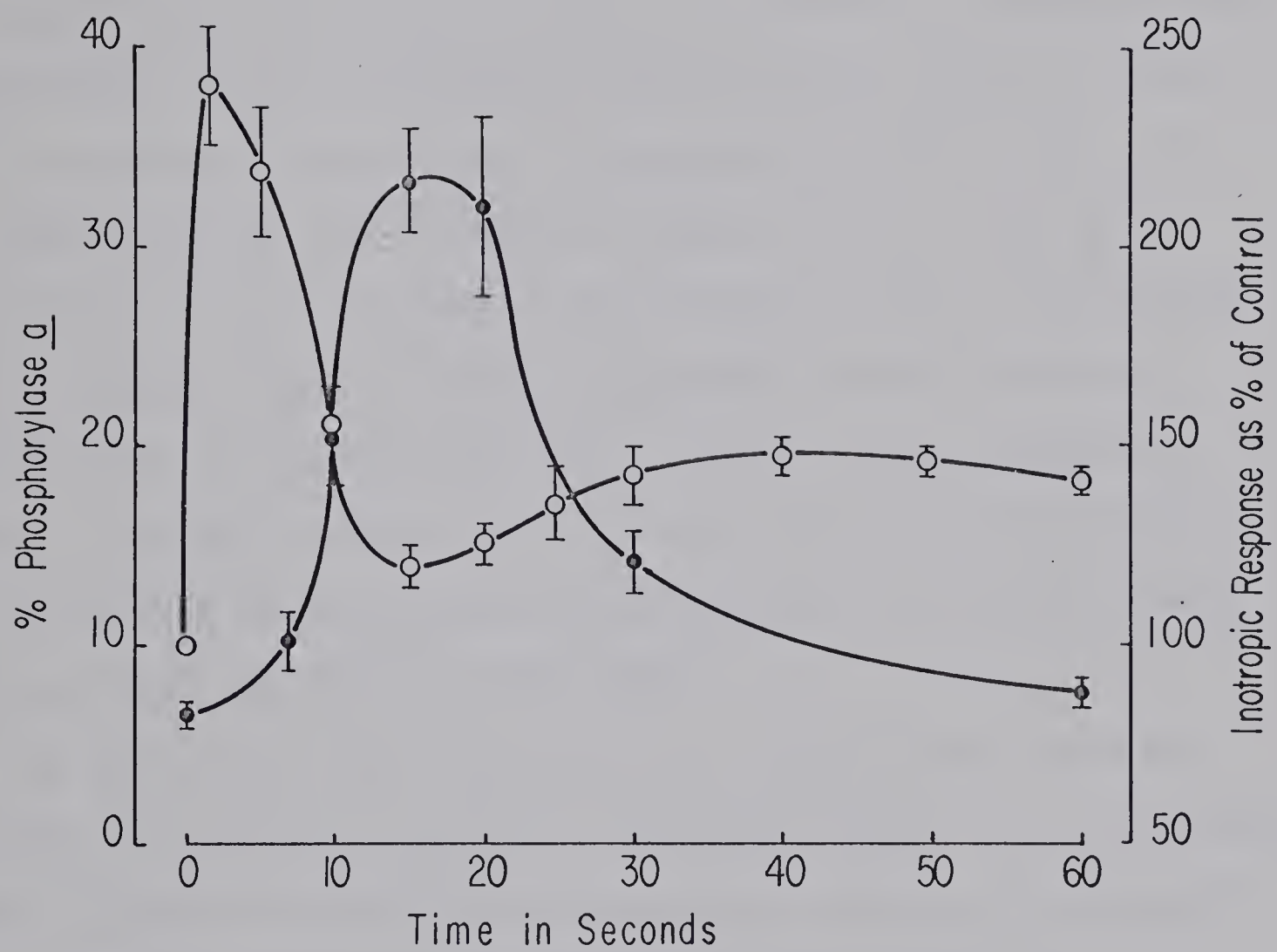
Figure 5 illustrates the results of the time-course study of the effects of paired-pulse stimulation on phosphorylase activation and positive inotropism.

It was observed that the systolic tension increased rapidly, reaching its peak (about 250% of control) within three seconds. The systolic tension then declined sharply, dipping nearly to its origin within 15 seconds, and finally rising again to level off at 150% of the original systolic tension.

Relative to the time for peak systolic tension to be developed, maximal phosphorylase activation was "late" by about 12 seconds. It is interesting that maximal phosphorylase activation occurs when systolic tension is declining, and when systolic tension is in fact, at its lowest ebb. If the absolute systolic tension is to be a direct measurement of intracellular free calcium ion concentrations, and maximal phosphorylase activation occurs at a time when systolic tension is at its lowest ebb, then it could be argued that: (a) a role for calcium ion in stimulating glycogenolysis through positive inotropism does not exist, or (b) the absolute systolic tension does not represent a true picture of the sarcoplasmic free calcium ion concentration.

Figure 5. Positive Inotropism and Phosphorylase Activation
Resulting from Continuous Paired-Pulse Stimulation

This figure illustrates the time-course effects of paired-pulse stimulation (60 msec delay) on phosphorylase activation (0—0) and the inotropic response (0—0). The time zero co-ordinate denotes control responses from 32 hearts, stimulated with single pulses (300/min) for the eight minute equilibration period (1.8 mM calcium Tyrode's). Paired pulse stimulation was begun at time zero, and an average of nine heart perfusions were done to obtain each experimental value. I-shaped bars symbolize standard errors of the means.



B. Abbreviated Periods of Paired-Pulse Stimulation

1. Objective

The results of the preceding experiment on the time-course study of positive inotropism in relation to phosphorylase activation has challenged the proposed theory of a role for calcium ion in glycogenolysis. Alternatively, it has been suggested that the absolute systolic tension does not represent a true picture of sarcoplasmic calcium levels. A considerable portion of the calcium necessary for stimulating glycogenolysis may in fact be released intracellularly during the later phase of the inotropic response to paired stimulation, possibly even while systolic tension is at its lowest ebb. A final, unlikely suggestion is that the systolic tension is a true representation of free calcium and that calcium ion does stimulate glycogenolysis, but that an inherent time-lag delays elicitation of this effect.

In order to test the validity of the foregoing suppositions an experiment was designed in which the time-course effects of an abbreviated period of paired stimulation on phosphorylase activation and inotropism were studied. It was felt that cessation of paired pulses at a time that was coincidental with the onset of the declining phase of the inotropic response would best serve this purpose. The net effect of this design was to retain the typical positive inotropic effect of paired pulses while, at the same time, accentuating the declining phase of the inotropic response. If, as suggested, a considerable portion of the

calcium necessary for stimulating glycogenolysis is being made available during the later phase (10-20 sec) of the inotropic response to continuous paired stimulation, then stopping paired-pulse stimulation at the onset of the declining phase should effectively reduce the calcium mobilization supposedly necessary for phosphorylase activation.

2. Experimental Design

Following the eight minute equilibration period (1.8 mM calcium), the isolated perfused rat hearts were switched from single to paired stimuli (60 msec delay) for seven seconds, then back to single pulses again. Tissue samples were taken at 0, 7, 12, 17, 22, 27, 47, and 87 seconds following the initiation of paired-pulse stimulation (first switch-over), and assayed for phosphorylase a. Measurements of the inotropic response were made directly and calculated as the "percent of control".

3. Results and Discussion

Figure 6 summarizes the results of the time-course study of the effects of seven seconds of paired-pulse stimulation on phosphorylase activation and positive inotropism. Referring again to Figure 5, it will be noted that seven seconds of paired stimulation is sufficient to elicit the typical maximal positive inotropic response normally seen with paired stimulation under these conditions. Upon

cessation of paired-pulse stimulation, systolic tension fell rapidly, but not immediately, to sub-control levels (50% of the control); after which it rose again and began to approach the control response that was normally obtained with single pulses.

Phosphorylase a levels continued to rise for ten seconds after the cessation of paired-pulse stimulation, but approached only one-half of the maximal value that had been obtained by stimulating continuously with paired pulses. After peak phosphorylase activity was reached, it rapidly declined and fell below control values (unpaired t test) within 40 seconds of cessation of paired pulses.

The results of these experiments indicate that a considerable portion of the intracellular calcium necessary for phosphorylase activation is being made available during the later phase (10-20 sec) of the inotropic response to continuous paired stimulation. This finding supports the suggestion that the absolute systolic tension may not represent the true picture of sarcoplasmic calcium levels during the inotropic response to paired-pulse stimulation. Also in view of the fact that the positive inotropic response to paired stimuli was left essentially intact by the abbreviated period of paired stimulation, while phosphorylase activation was reduced by more than one-half, it seems unlikely that a time-lag factor of more than five seconds could be involved in the mechanism of calcium-induced phosphorylase activation.

In summary, it is suggested that the positive inotropic

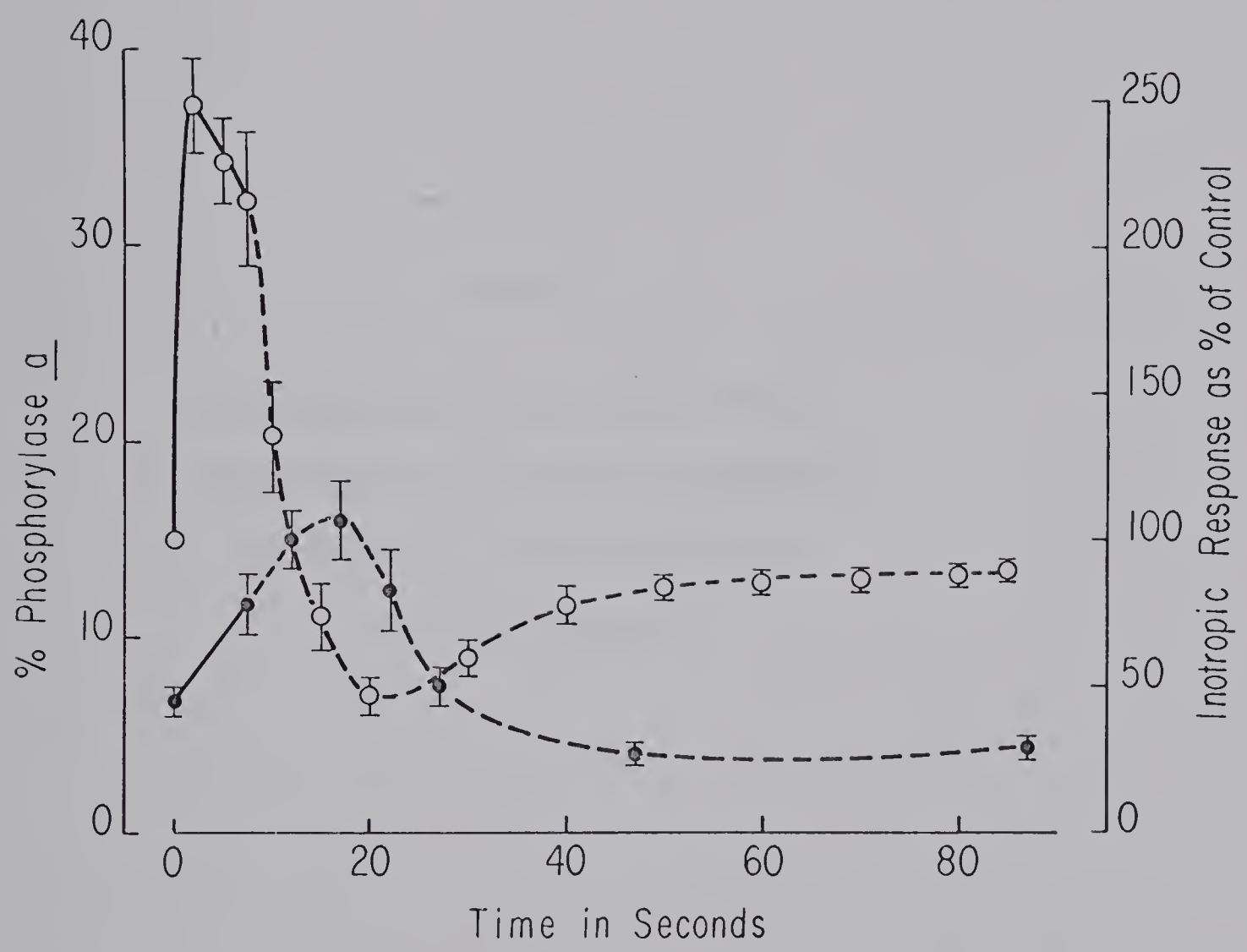
response does indicate that calcium has been mobilized and that intracellular free calcium levels were raised^(19,20,53,56,57).

However, it is further suggested that not all of the calcium being made available in this way is being mobilized during the development of peak systolic tension, but that a very considerable portion of the calcium made available comes during the declining phase of the response, and even while the declining phase is at its lowest ebb.

Further evidence supporting the above suggestions is given in Chapters VII and VIII.

Figure 6. Positive Inotropism and Phosphorylase Activation
 Resulting from an Abbreviated Period of Paired-
 Pulse Stimulation

This figure illustrates the time-course effects of seven seconds of paired-pulse stimulation (solid lines) followed by a return to single pulse stimulation (broken lines) on phosphorylase activation (closed circles) and the inotropic response (open circles). The time-zero co-ordinate denotes control responses from 32 hearts, stimulated with single pulses (300/min) for the eight minute equilibration period (1.8 mM calcium Tyrode's). Paired pulse stimulation (60 msec delay) was begun at time zero, and an average of nine heart perfusions were done to obtain each experimental value. I-shaped bars symbolize standard errors of the means.



CHAPTER V

Inhibition of Paired Pulse-Induced Phosphorylase Activation by Abruptly Lowering the Perfusing Calcium Ion Concentration

A. Introduction and Objective.

Since Friesen et al. (1969)⁽⁵⁰⁾ have established that an abrupt three-fold increase in the perfusing calcium ion concentration causes an activation of phosphorylase that is associated with positive inotropism, it was deemed of interest to study what effects an abrupt decrease in the perfusing calcium ion concentration would have on inotropism and/or phosphorylase activation. In the two previous chapters, experiments were described in which paired-pulse stimulation was used to produce an increase in the force of myocardial contraction and phosphorylase a levels. In this chapter, experiments will be described in which the perfusing calcium ion concentration was abruptly lowered in an attempt to prevent these responses to paired-pulse stimulation.

B. Experimental Design.

Following the eight minute equilibration period with single pulses (300/min), using a 2.4 mM calcium Tyrode's solution, the perfusion was abruptly changed for one containing 0.6 mM calcium. Perfusion with this latter solution was continued for the remainder of the experiment. Stimulation with paired pulses (60 msec delay) was initiated 15 seconds after the switch to the solution containing 0.6 mM calcium, this particular time sequence was found to be the best for demonstrating inhibition of paired pulse-induced positive

inotropism by the low-calcium perfusion.

Tissue samples were taken at 0, 10, 15, and 20 seconds after the initiation of paired stimulation and assayed for phosphorylase activity.

C. Results.

The results of these experiments are compared with those obtained in hearts where the perfusing calcium ion concentration was not lowered (Chapter IV, A). It should be noted here that the comparison made is between paired pulses at 1.8 mM calcium, and paired pulses following a switch from 2.4 to 0.6 mM calcium. A change from 1.8 to 0.6 mM calcium was not used because it was felt that a greater inhibitory effect on phosphorylase activation and positive inotropism would be shown if the perfusing calcium level was abruptly lowered over a larger range. It is relevant to emphasize at this point, that in terms of reference to inotropism in this Chapter, and in the following Chapters, inotropism shall be expressed and referred to in absolute terms (grams systolic tension), and not relative terms (percent of control). As pointed out earlier (Chapter II, D.), absolute measurements of inotropic changes may better reflect the intracellular free calcium ion concentration during systole.

Figure 7 shows typical tracings of the isometric contractile responses that were obtained using the above described parameters separately and in combination. Abruptly lowering the perfusing

calcium ion concentration from 2.4 to 0.6 mM resulted in a negative inotropic response, as was evidenced by a fall in the systolic tension. Paired-pulse stimulation of hearts equilibrated with 1.8 mM calcium Tyrode's resulted in the rapid production of a typically transient, positive inotropic response. When paired pulses were administered to hearts 15 seconds after the perfusing calcium ion concentration had been abruptly lowered (as described), the typical positive inotropic response was not seen. Both the rate of onset and the rate of offset (declining phase) of positive inotropism were markedly slowed, and the maximal systolic tension that was developed did not generally exceed the normal control response that had been observed before the switch to low calcium. It was concluded that the abrupt switch to low calcium had prevented the development of maximal systolic tension usually obtained with paired-pulse stimulation.

Figure 8 shows how the abrupt four-fold decrease in the perfusing calcium ion concentration affected the ability of paired pulses to cause phosphorylase activation. As previously described, stimulation with paired-pulses can cause a rise in phosphorylase a levels to about 30% within 15 seconds (1.8 mM calcium). When the concentration of calcium ions in the perfusion fluid was abruptly lowered, the ability of paired pulses to activate phosphorylase was completely abolished. The statistical significance was tested using the unpaired t test.

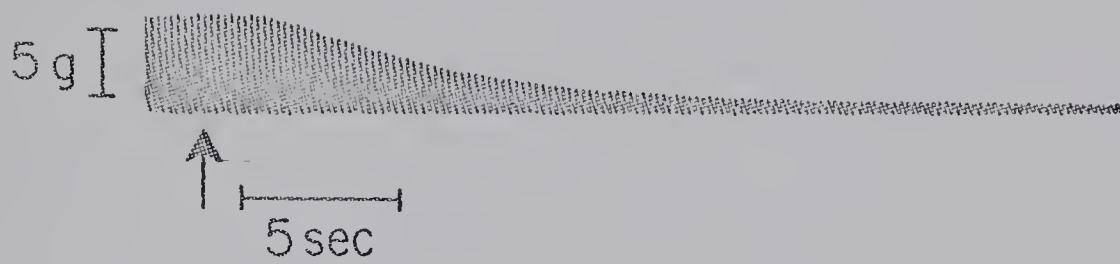
Control experiments were done which showed that there is no statistically significant difference in basal phosphorylase a values in hearts equilibrated with 2.4, 1.8, or 0.6 mM calcium Tyrode's solutions. It was also shown that an abrupt lowering of the perfusing calcium ion concentration from 2.4 to 0.6 mM does not affect basal phosphorylase a levels.

These experiments demonstrate that abruptly lowering the concentration of perfusing calcium ions can: (1) markedly inhibit the systolic tension (in absolute terms), and (2) completely abolish the increase in phosphorylase activation than is normally induced by stimulation with paired pulses. This data provides evidence to support the role for calcium ion in phosphorylase activation, implicating that because development of maximal systolic tension was prevented by the abrupt switch to low calcium, insufficient sarcoplasmic free calcium was made available for causing phosphorylase activation.

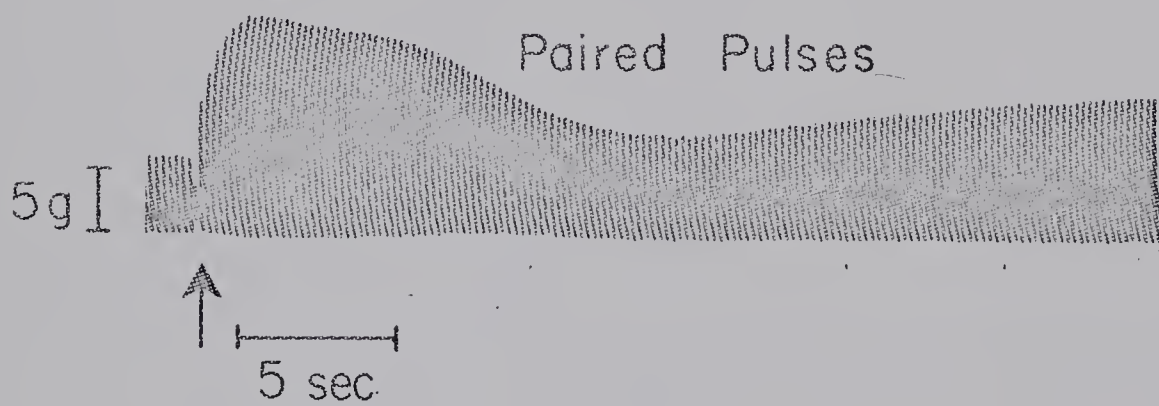
Figure 7. Inhibition of Paired Pulse-Induced Positive
Inotropism by an Abruptly Lowered Calcium
Ion Concentration in the Perfusion

Shown here are typical tracings of the isometric contractile responses obtained. Top tracing: abruptly lowering the calcium ion concentration from 2.4 to 0.6 mM. Center tracing: paired pulse stimulation (60 msec delay) following equilibration with 1.8 mM calcium. Bottom tracing: paired pulse stimulation 15 seconds after abruptly lowering the calcium ion concentration from 2.4 to 0.6 mM. All responses were elicited after the eight minute equilibration period with single pulse stimulation (300/min).

Low $[Ca^{++}]$



Paired Pulses



Low $[Ca^{++}]$ Paired Pulses

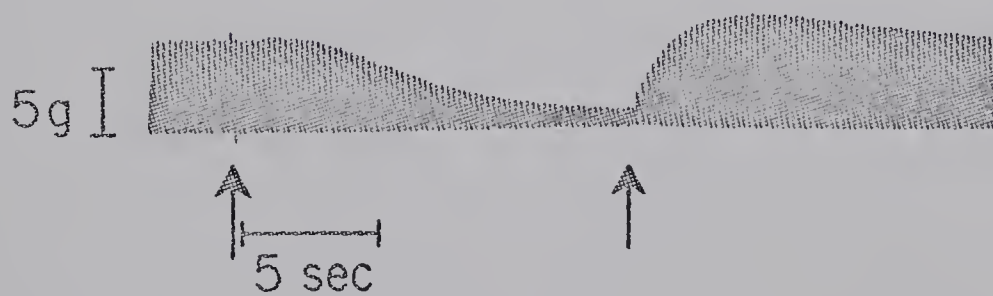
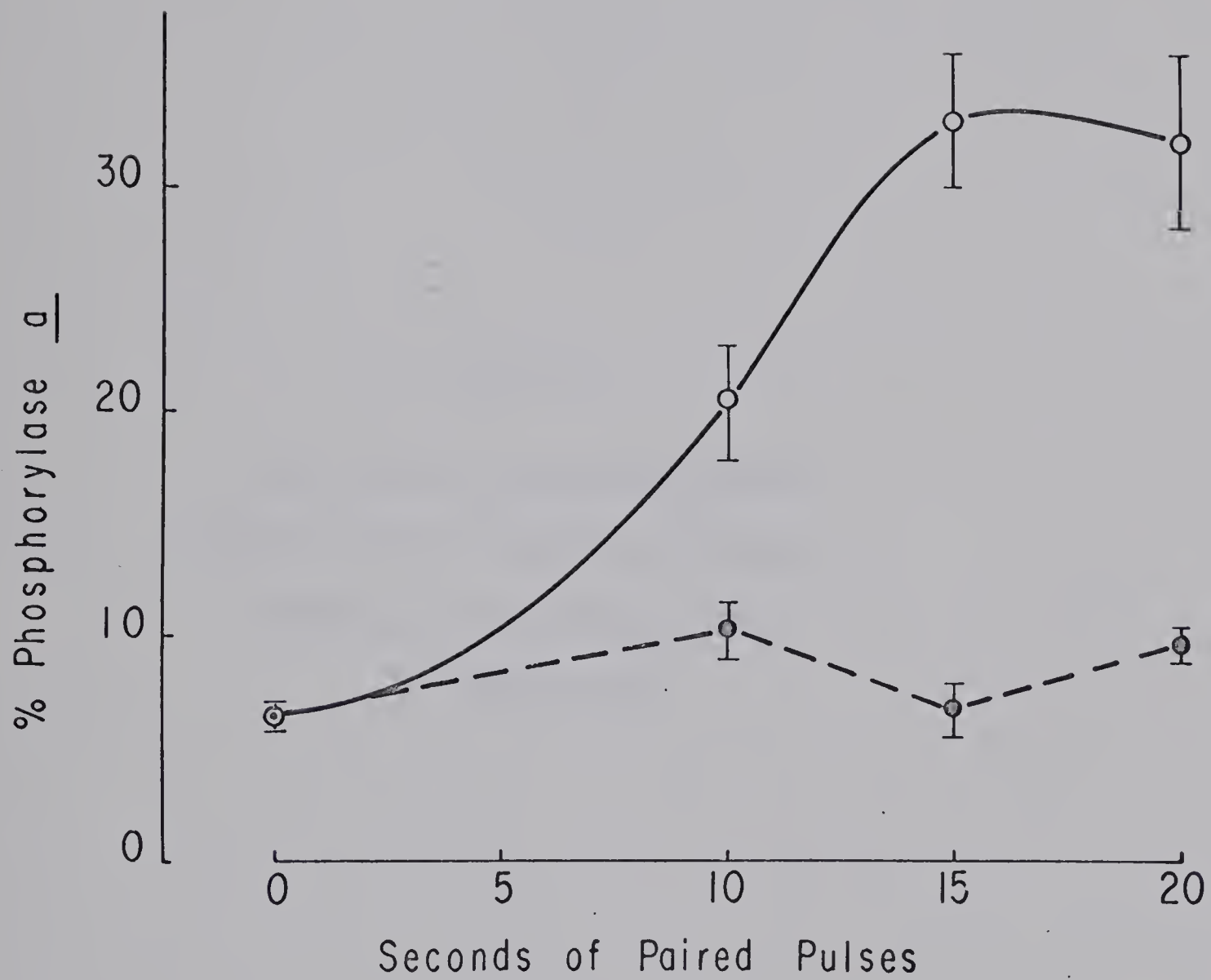


Figure 8. Inhibition of Paired Pulse-Induced Phosphorylase
Activation by an Abruptly Lowered Calcium
Ion Concentration in the Perfusion

Plotted are the time-course effects of paired pulse stimulation (60 msec delay) on phosphorylase activation, following equilibration with 1.8 mM calcium Tyrode's (0—0). These effects are compared with those obtained when the perfusing calcium ion concentration was abruptly lowered, from 2.4 to 0.6 mM, fifteen seconds prior to the stimulation with paired pulses (0--0). The time zero co-ordinate denotes the mean (control) phosphorylase values obtained from 32 hearts equilibrated for 8 minutes at 1.8 mM calcium; and 4 hearts equilibrated for an 8 minute period with 2.4 mM calcium, followed by 15 seconds of 0.6 mM calcium perfusion. Paired pulse stimulation was begun at time zero, and an average of seven heart perfusions were done to obtain each experimental value. I-shaped bars symbolize standard errors of the means.



CHAPTER VI

Inhibition of Epinephrine-Induced
Phosphorylase Activation by Abruptly
Lowering the Perfusing Calcium
Ion Concentration

A. Introduction and Objective.

The role of catecholamines in glycogen phosphorylase activation has been well established (see Chapter I). More recently it has been demonstrated that calcium ion plays an obligatory role in catecholamine-induced activation of phosphorylase, possibly by permitting activated phosphorylase b kinase to convert phosphorylase b to phosphorylase a⁽⁴⁹⁾. However, the degree to which catecholamine-induced phosphorylase activation depends on the calcium that is mobilized during positive inotropism, has not been fully investigated. Namm et al.⁽⁴⁹⁾ have shown that perfusion without calcium causes the phosphorylase-activating effect of epinephrine administration to be "greatly diminished but not abolished" (55% reduction).

In the experiments to be described in this chapter, an attempt was made to establish the degree to which epinephrine-induced phosphorylase activation depends upon the typical positive inotropic response that is normally elicited following administration of this hormone. It will be recalled, that increases in myocardial contractility are associated with increased mobilization of calcium ions^(19,20). For this purpose experiments were designed in which an abrupt, four-fold decrease in the perfusing calcium ion concentration was used to counteract the positive inotropic effect of a given dose of epinephrine. The objective was to prevent the normal, positive inotropic response to

epinephrine, thereby determining to what extent this response per se is necessary for phosphorylase activation by catecholamines.

B. Experimental Design.

Following the eight minute equilibration period with single pulses (300/min), using 2.4 mM calcium Tyrode's solution, the perfusing Tyrode's solution was abruptly changed for one containing 0.6 mM calcium. Administration of epinephrine (0.25 μ g over a 5-sec period, as previously described) was initiated at the same time as the switch to low-calcium Tyrode's; this particular time sequence was found to be the best for demonstrating inhibition of epinephrine-induced positive inotropism.

Tissue samples were taken at 0, 10, 15, 20, and 25 seconds after beginning epinephrine injection, and assayed for phosphorylase a.

For comparative purposes, a control time-course study of the phosphorylase-activating effect of epinephrine was also done. The same procedure as described above was followed, but the calcium ion concentration was maintained at 2.4 mM throughout the perfusion period.

C. Results and Discussion

Figure 9 shows typical tracings of the isometric contractile responses that were obtained by epinephrine administration alone,

and epinephrine administration simultaneous with an abruptly lowered perfusing calcium ion concentration. When both epinephrine and the low-calcium perfusion were administered, the positive inotropic response to epinephrine was first preceded by a negative inotropic response to the low-calcium (see also Figure 7). The net result of this interaction was a reduction in the maximal systolic tension developed, with a slower rate of onset.

It is of significance to point out here that partial contracture (increased end-diastolic tension), which is often seen with maximal positive inotropism (e.g.: see epinephrine alone, declining phase Figure 9), was also prevented by the abrupt switch to low calcium (0.6 mM). Partial contracture, and its implications, shall be discussed further in Chapter IX.

In terms of phosphorylase activation, the time-course effect of epinephrine alone and epinephrine plus low calcium are illustrated in Figure 10. Peak phosphorylase a levels were 40% lower (30% vs 70%) with epinephrine in the presence of sudden 0.6 mM calcium, than with epinephrine alone and under control conditions (2.4 mM calcium Tyrode's). This represents a reduction of more than one-half. These results suggest that an abruptly lowered calcium ion concentration in the perfusion by effectively preventing a maximal positive inotropic response to epinephrine, is effective in reducing the degree of phosphorylase activation obtained. This finding implies that the above procedure reduces the role of calcium ion in stimulating glycogenolysis. Therefore, a considerable portion of the phosphorylase activation remaining

may be due to the calcium independent, adenyl cyclase-mediated activation of phosphorylase b kinase^(18,49). It will be appreciated however, that the accuracy of such a statement depends on the degree of success with which one is able to administer epinephrine to a normally contracting heart without changing its contractile response. Further experimentation along these lines is intended.

Figure 9. Inhibition of Epinephrine-Induced Positive
Inotropism by an Abruptly Lowered Calcium
Ion Concentration in the Perfusion

Shown here are typical tracings of the isometric contractile responses obtained. Top tracing: Administration of epinephrine (0.25 μ g over a 5-sec interval) following equilibration with 2.4 mM calcium. Bottom tracing: Administration of epinephrine simultaneous to abruptly lowering the perfusing calcium ion concentration from 2.4 to 0.6 mM. All responses were elicited after the eight minute equilibration period with single pulse stimulation (300/min).

The apparent "negative inotropism" preceding the positive inotropic effect of epinephrine alone (top tracing) is atypical, and may therefore be disregarded as being of any consequence.

EPI

5g I

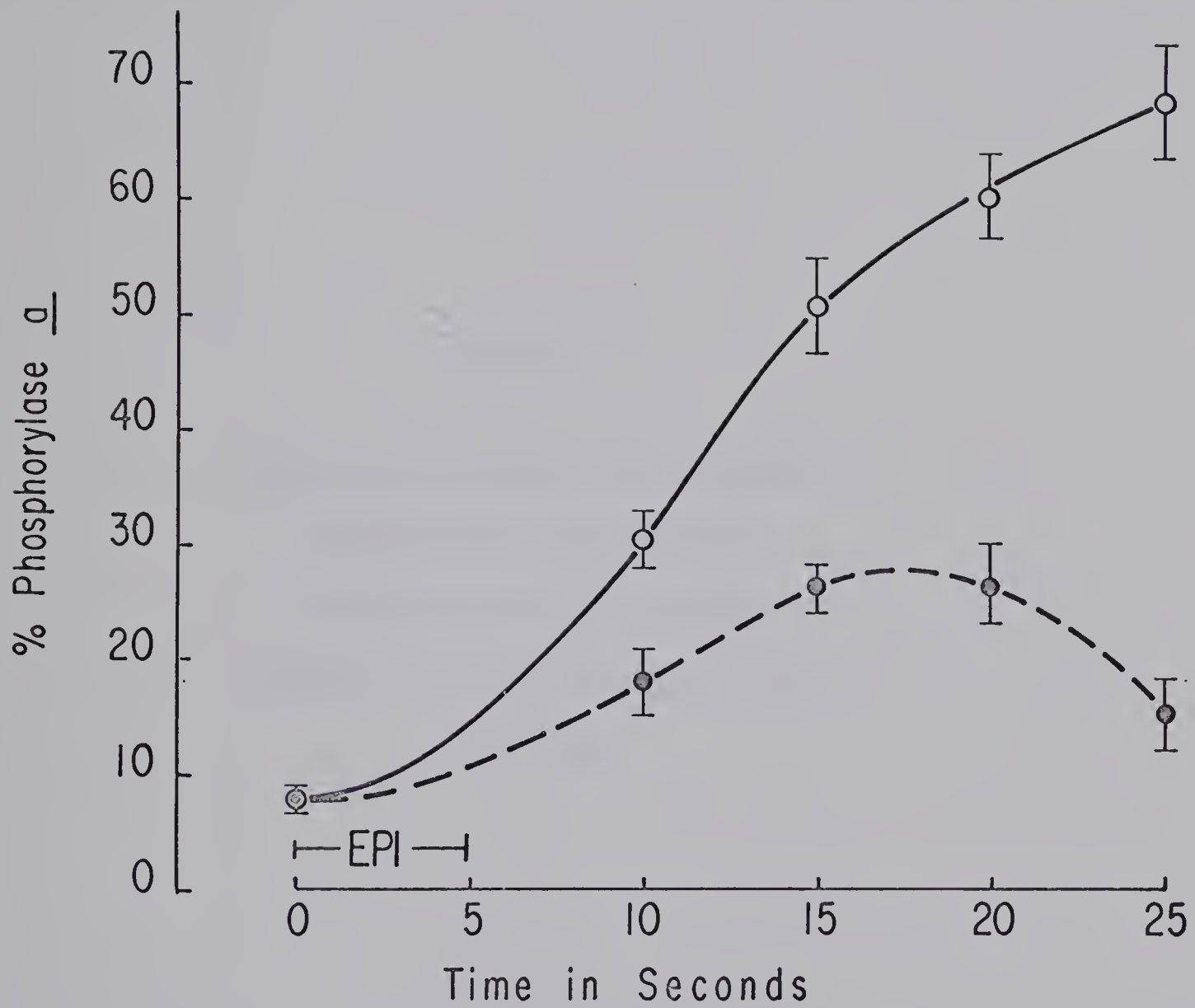


5 sec

EPI + Low $[Ca^{++}]$ 

Figure 10. Inhibition of Epinephrine-Induced Phosphorylase
Activation by an Abruptly Lowered Calcium Ion
Concentration in the Perfusion

Plotted are the time-course effects of epinephrine administration (0.25 μ g over a 5-sec interval) on phosphorylase activation, following equilibration with 2.4 mM calcium Tyrode's (0—0). Also plotted are the time-course effects that were observed when the perfusing calcium ion concentration was abruptly lowered, from 2.4 to 0.6 mM, simultaneous to the administration of epinephrine (0--0). The time-zero co-ordinate denotes control phosphorylase values obtained from 29 hearts, stimulated with single pulses (300/min) for the eight minute equilibration period. Epinephrine administration was begun at time zero, and an average of seven heart perfusions were done to obtain each experimental value. I-shaped bars symbolize standard errors of the means.



CHAPTER VII

Inhibition of Paired-Pulse Induced
Phosphorylase Activation by
Equilibrating with Decreased
Calcium Ion Concentrations in the
Perfusion

A. Objective.

The objective of this series of experiments was to discover whether or not equilibrating the isolated, perfused rat hearts with decreased calcium ion concentrations would be effective in inhibiting the positive inotropic response and/or phosphorylase activation normally seen with paired pulse-stimulation.

B. Experimental Design.

Following eight minutes equilibration with 0.6, 1.2, or 1.8 mM calcium Tyrode's solution, the isolated perfused rat hearts were switched from single to paired-pulse stimulation (300/min, 60 msec delay). Tissue samples were taken at 0, 10, 15, 20, 25 and/or 30 seconds after initiation of paired-pulse stimulation and assayed for phosphorylase a.

C. Results and Discussion.

As shown in Figure 11, equilibration with decreased calcium ion concentrations was effective in inhibiting paired pulse-induced activation of phosphorylase. The degree of inhibition was increased by decreasing the concentration of calcium ions used.

As found and stated previously, equilibration with different calcium ion concentrations produced no statistically significant

change in the basal control levels of phosphorylase a. In fact Namm et al. ⁽⁴⁹⁾ have shown, under slightly different conditions, that perfusion with zero-calcium Tyrode's does not change basal phosphorylase a levels.

The positive inotropic response to paired pulse stimulation, following equilibration at decreased calcium ion concentrations, was markedly affected. The maximal systolic tension developed was not reduced by a statistically significant degree. However, the rate of onset of positive inotropism was greatly slowed. This effect was readily measured as the time required for the positive inotropic response to develop maximal (peak) systolic tension (Table 2), and could not simply be accounted for by the slight reduction in systolic tension which accompanies equilibration at lowered calcium ion concentrations. Concomitant with a slowed rate of onset of positive inotropism was a slowed rate of offset (declining phase). In those cases where the calcium ion concentration employed during equilibration had been decreased, and the rates of onset and offset of positive inotropism were slowed; no increase in the end-diastolic tension occurred. Such an increase in end-diastolic tension (partial contracture) is often associated with the declining phase (offset) of a maximal positive inotropic response.

These results suggest that equilibration of perfused hearts with a decreased calcium ion concentration is effective in inhibiting

paired pulse-induced phosphorylase activation. It was concluded that this inhibition does not correlate as well to a reduced maximal systolic tension, as it does to a slowing in the rate of onset of maximal positive inotropism. A further discussion of the reasons for this conclusion is given in Chapter IX.

Figure 11. Inhibition of Paired Pulse-Induced Activation
of Phosphorylase by Equilibration with
Decreased Calcium Ion Concentrations

Shown here are the time-course effects of paired pulse stimulation (60 msec delay) on phosphorylase activation, following equilibration with 1.8 mM calcium Tyrode's ($\square-\square$); 1.2 mM calcium Tyrode's ($0-0$); and 0.6 mM calcium Tyrode's ($0-0$). The time-zero co-ordinate denotes control phosphorylase values obtained from 49 hearts, stimulated with single pulses (300/min) for the eight minute equilibration period. Paired pulse stimulation was begun at time zero, and an average of seven heart perfusions were done to obtain each experimental value. I-shaped bars symbolize standard errors of the means.

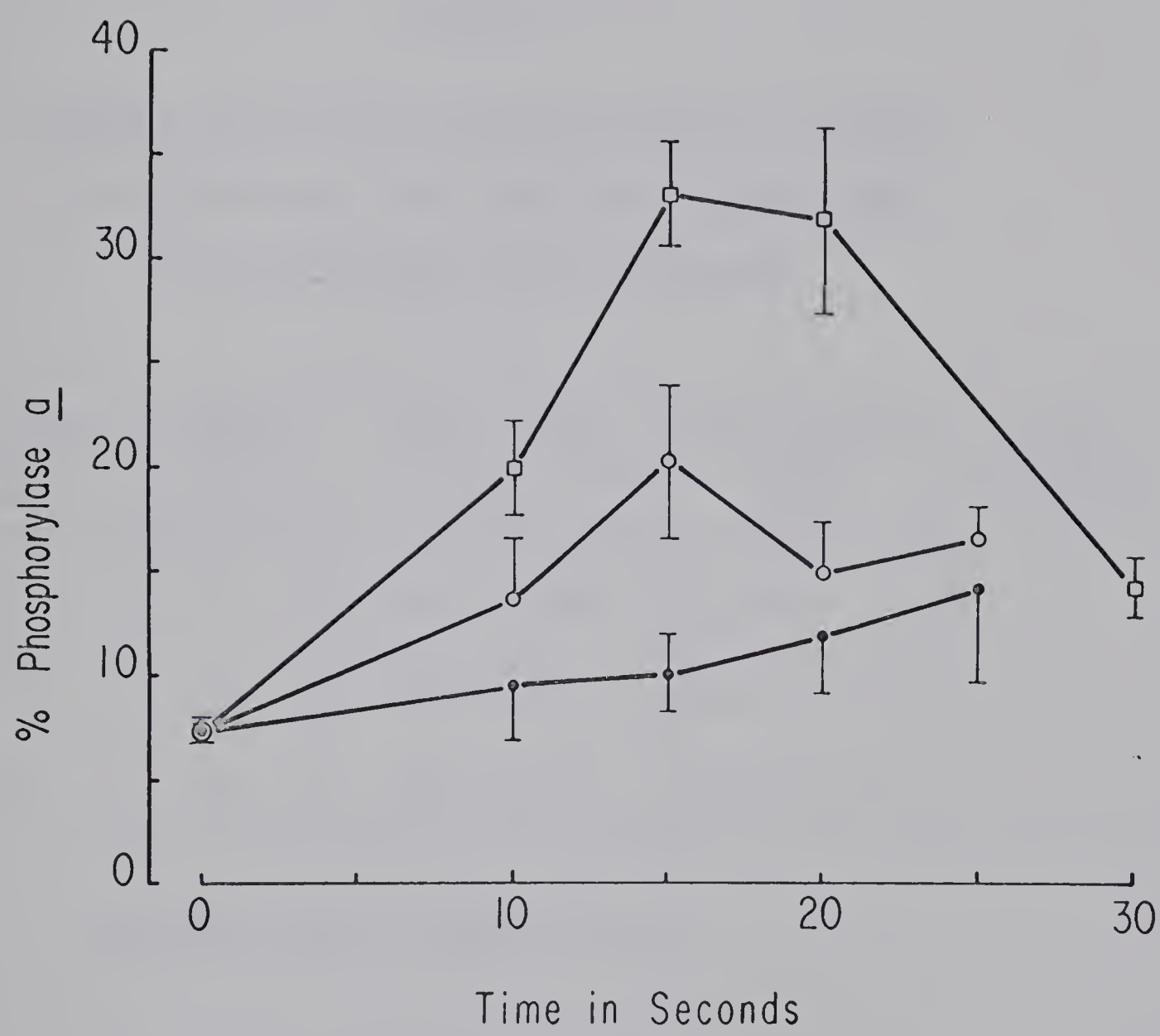


TABLE II

Changes in the Positive Inotropic Response to Paired
Pulse Stimulation After Equilibrating Hearts With
Different Calcium Ion Concentrations

Calcium Ion Concentration (mM)	Number of Samples	Time to Peak Systolic Tension (sec)	Peak Systolic Tension (g)	Partial Contracture Evident
0.6	8	7.15 ± 0.28^a	14.3 ± 0.7	—
1.2	6	3.80 ± 0.27	15.2 ± 0.9	—
1.8	6	2.00 ± 0.20	16.2 ± 1.2	+

^a Represents mean $\pm$ standard error.

CHAPTER VIII

Inhibition of Epinephrine-Induced Phosphorylase Activation by Equilibrating with Decreased Calcium Ion Concentrations in the Perfusion

A. Objective.

The object of this series of experiments was to discover whether or not equilibrating the isolated perfused rat hearts with decreased calcium ion concentrations would be effective in inhibiting the positive inotropic response and/or phosphorylase activation normally seen with epinephrine administration. Work done by Ellis and Vincent⁽⁵⁵⁾ tended to indicate that little or no inhibition would be seen. These workers have shown that a five-fold reduction (2.5 mM to 0.5 mM) in the calcium ion concentration of K-H-B solution perfusing isolated guinea pig hearts at 27°C, does not effectively reduce the epinephrine-induced activation of phosphorylase seen in normal K-H-B with 2.5 mM calcium.

B. Experimental Design.

Following eight minutes equilibration with single pulses (300/min), and using either 0.6, 0.9, 1.2, or 2.4 mM calcium Tyrode's solution; the isolated, perfused rat hearts were injected with epinephrine (0.25 µg over 5-sec period) as previously described. Tissue samples were taken at 0, 10, 15, 20, 25, and 30 seconds after starting the epinephrine infusion and assayed for phosphorylase a.

C. Results and Discussion.

As shown in Figure 12, equilibration with decreased calcium

ion concentrations was effective in inhibiting epinephrine-induced activation of phosphorylase. The degree of inhibition was increased by decreasing the concentration of calcium used.

Alterations in the positive inotropic response to epinephrine, following equilibration at decreased calcium ion concentrations, were similar to those described in Chapter VII (paired stimulation). Namely, the rates of onset and offset of maximal positive inotropism were markedly slowed, and no statistically significant differences in the maximal systolic tensions developed with 2.4, 1.2, and 0.9 mM calcium Tyrode's were noticed. Equilibration with 0.6 mM calcium Tyrode's, however, was an exception in that the maximal positive inotropic response to epinephrine was significantly reduced (Table 3). Partial contracture was only observed following epinephrine administration to hearts which had been equilibrated with 2.4 mM calcium. The relevance of this observation shall be discussed later.

It was concluded that equilibration of perfused hearts with a decreased calcium ion concentration is effective in inhibiting epinephrine-induced phosphorylase activation. It seemed apparent that this inhibition does not correlate as well to a reduced peak systolic tension, as it does to a slowing of the rate of onset of maximal positive inotropism; although the former effect maybe somewhat modified by the latter.

Discussion of the inotropic response and its correlation to phosphorylase activation is undertaken in greater detail in the following chapter.

Figure 12. Inhibition of Epinephrine-Induced Activation
of Phosphorylase by Equilibration with
Decreased Calcium Ion Concentrations

Shown here are the time-course effects of epinephrine administration (0.25 μ g over a 5-sec interval) on phosphorylase activation, following equilibration with 2.4 mM calcium Tyrode's ($\blacksquare$ — $\blacksquare$); 1.2 mM calcium Tyrode's ($\square$ — $\square$); 0.9 mM calcium Tyrode's (0—0); and 0.6 mM calcium Tyrode's ($\circ$ — $\circ$). The time-zero co-ordinate denotes control phosphorylase values obtained from 48 hearts, stimulated with single pulses (300/min) for the eight minute equilibration period. Epinephrine administration was begun at time zero, and an average of seven heart perfusions were done to obtain each experimental value. I-shaped bars symbolize standard errors of the means.

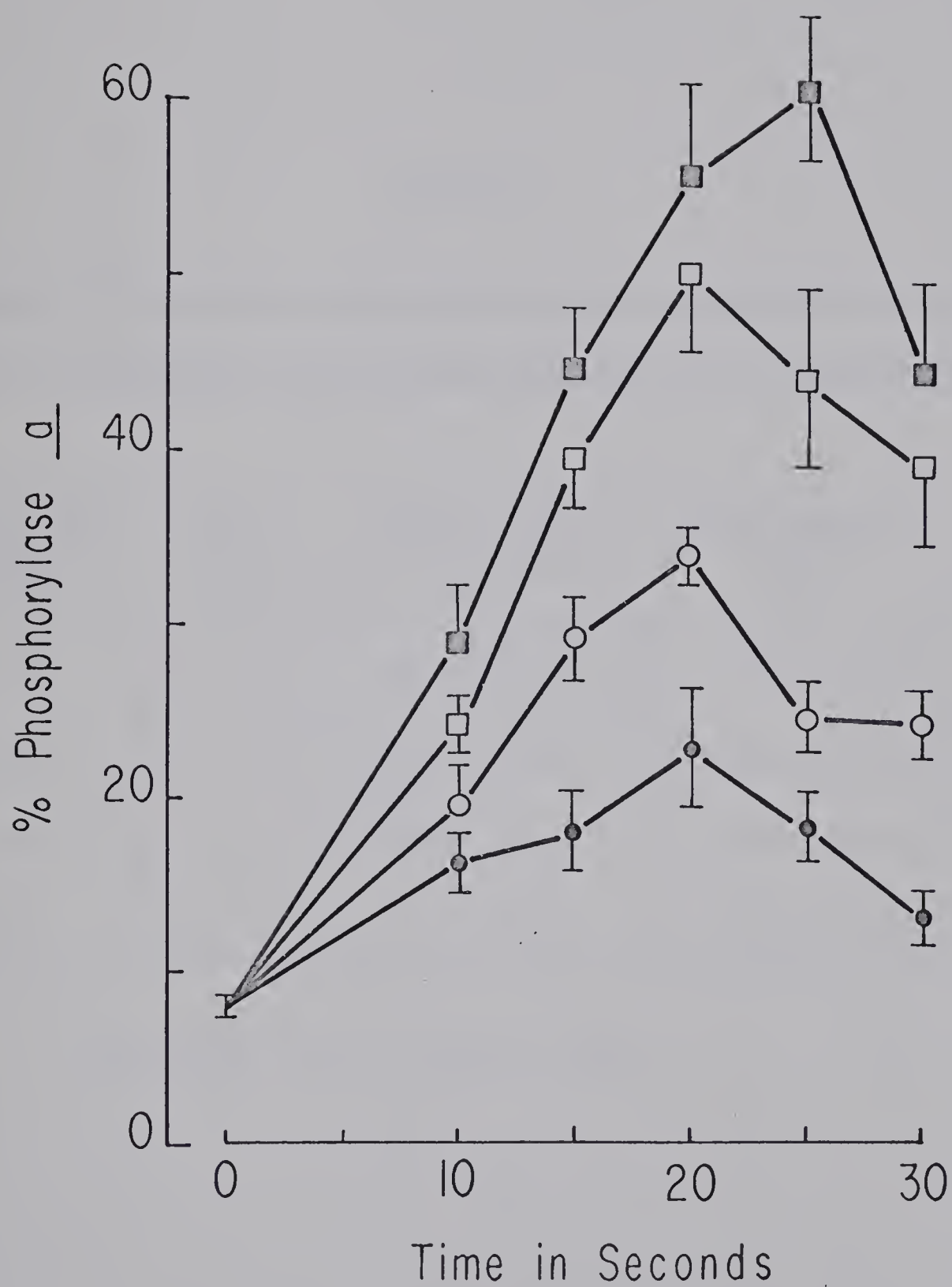


TABLE III

Changes in the Positive Inotropic Response to Epinephrine after
Equilibrating Hearts with Different Calcium Ion Concentrations

Calcium Ion Concentration (mM)	Number of Samples	Time to Peak Systolic Tension (sec)	Peak Systolic Tension (g)	Partial Contracture Evident
0.6	15	23.2 ± 0.3^a	11.8 ± 0.8	—
0.9	12	19.4 ± 0.6	14.6 ± 0.6	—
1.2	14	18.0 ± 0.7	15.0 ± 0.5	—
2.4	8	15.2 ± 0.7	14.0 ± 0.8	+

^a Represents mean $\pm$ standard error.

CHAPTER IX

An Attempt to Correlate Positive Inotropism with Phosphorylase Activation

The results of the foregoing experiments suggest that a positive inotropic response that is rapid in onset and approaching maximal systolic tension (14 g under these conditions), may be associated with phosphorylase activation.

For normal cardiac function to be maintained, each contraction must be followed by a relaxation. After a series of rapidly induced "maximal" contractions it is possible that calcium could accumulate intracellularly so as to partially swamp the relaxing mechanism. This might be accompanied by a slowing of the process by which free intracellular calcium is sequestered. If intracellular free calcium levels were thus "elevated" over prolonged periods of time, relaxation might be slower, and partial contraction might become evident. Slowed relaxation and partial contracture did occur (Figure 9). The systolic tension declined during this phase of the positive inotropic response. This indicates that less calcium was available for release into the sarcoplasm. Implicit in this observation is the understanding that the relaxing mechanism and calcium releasing mechanism are not one and the same. It is suggested then, that although the absolute maximal systolic tension developed may represent the peak free calcium ion concentration in the region of the contractile proteins during a single contraction, it does not necessarily represent a true picture of free calcium ion concentrations integrated over time. Free calcium levels may be just as "high" during the declining phase of inotropic response

as during the peak phase. Electron microscopy has shown that the glycogen granules are located in the cytoplasm between the sarcomembrane and the contractile proteins^(60,61,62). Phosphorylase b and a small portion of phosphorylase b kinase has been found to be bound to the glycogen granules⁽⁶³⁾. Phosphorylase activation must therefore occur in this region of the cytoplasm, and any role for calcium ion in stimulating the activity of phosphorylase b kinase must necessarily depend on the concentration of free calcium (as a function of time) in this region. If calcium-for-contraction must diffuse from the sarcomembrane to the region of the contractile proteins, it must first cross the region of the glycogenolytic enzymes. The special differentiation of these two regions may be an important factor in determining the free calcium levels attained by each of these regions during a positive inotropic response, and therefore the concentration of calcium (as a function of time) made available to the glycogenolytic enzymes. A diagramatic representation of the foregoing hypothesis is shown in Figures 13 and 14, which illustrate the operative cellular components in relation to the author's concept of calcium ion mobility and concentration.

Emphasis is placed on the fact that the positive inotropic response must be induced rapidly. It was noted in Chapters VII and VIII that although maximal systolic tension was always evoked by the stimulatory parameter (paired pulses or epinephrine), at lower equilibrating calcium

ion concentrations the rate of onset of positive inotropism was slower, (It is possible that a slower rate of onset is the result of a reduced availability of calcium-for-release.) Because the rate of onset is slowed, it is suggested that the metabolic processes controlling internal calcium uptake and release are given time to adjust, and swamping of the calcium uptake system does not occur. Hence, elevation of intracellular free calcium levels (as integrated over time) may be somewhat less and phosphorylase activation is consequently reduced.

Figure 13. Myocardium

Illustrated on the opposite page is a sketch of small section of myocardial cell. This drawing was taken from a number of electron-micrograph plates^(60,61,62). Abbreviations used are:

CPR = Contractile Protein Region, GER = Glycogenolytic Enzyme Region, ECF = Extracellular Fluid, SM = Sarcomembrane, G = Glycogen granules, and M = Mitochondrion. (See also Figure 14.)

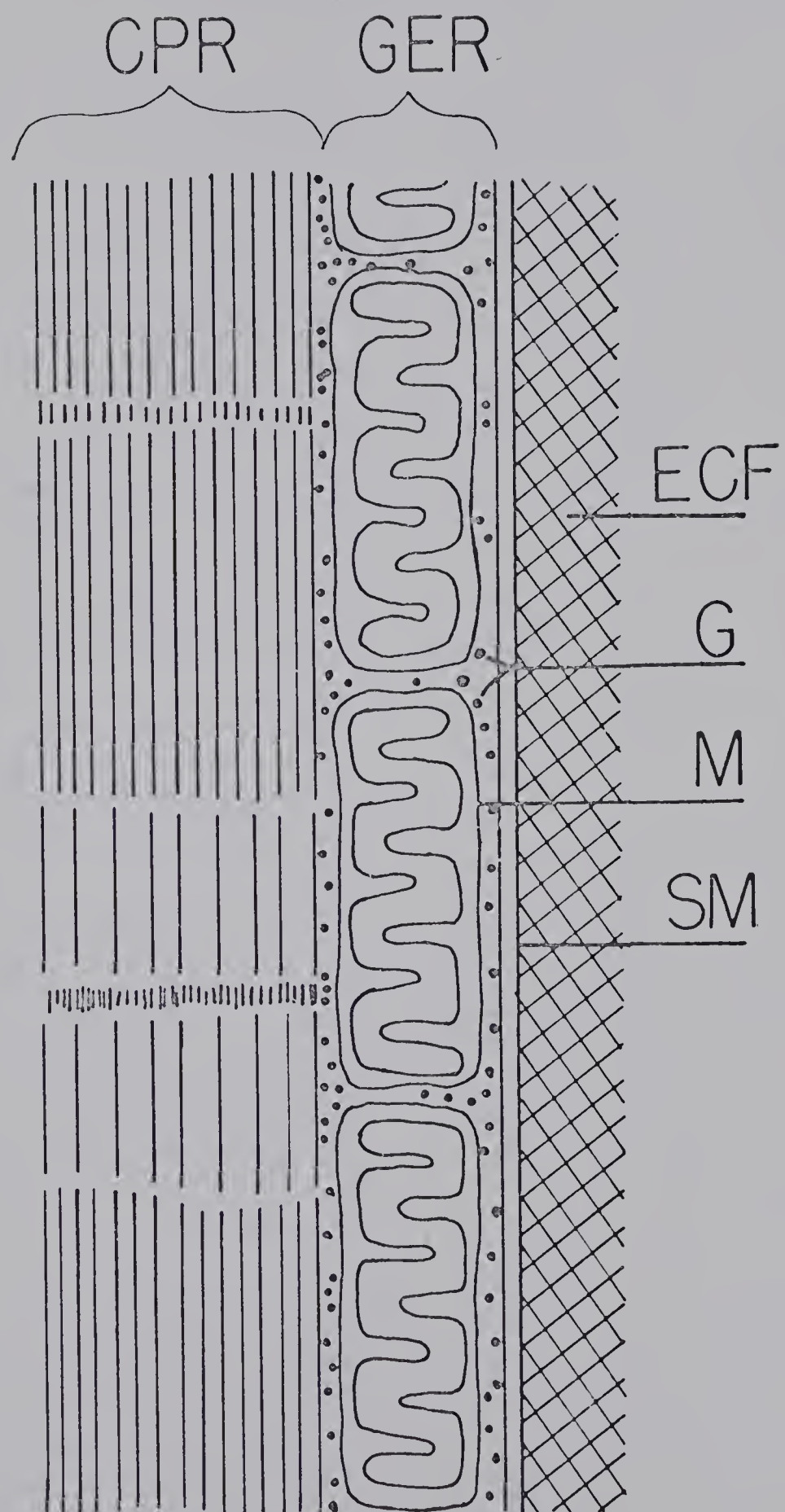


Figure 14. Intracellular Calcium Ion Concentrations
 as a Function of Time

This model shows how myocardial intracellular calcium ion levels, if integrated over time, could be relatively high in the region of the glycogenolytic enzymes (GER, bottom), even though the systolic force of contraction is decreasing as the result of decreased peak calcium ion concentrations in the region of the contractile proteins (CPR, top; see also Figure 13). Such a positive inotropic response could be induced by paired pulses (PP). More contractions than are shown here would normally be involved.

This model assumes the following: (1) that the calcium for contraction is released from the sarcomembrane, (2) that the calcium uptake site is in the region of the sarcomembrane, (3) that calcium uptake and release sites are not one and the same, (4) that the uptake mechanism becomes saturated before it can accommodate for the increased load, thereby producing partial contracture and slowed relaxation, (5) that calcium-for-release becomes depleted and results in decreasing peak calcium concentrations during the declining phase, (6) that the greatest amount of free calcium integrated over time is made available not more than five seconds before peak phosphorylase activation, and (7) that calcium release and uptake mechanisms accommodate for the increased load and equilibration at steady state levels results.

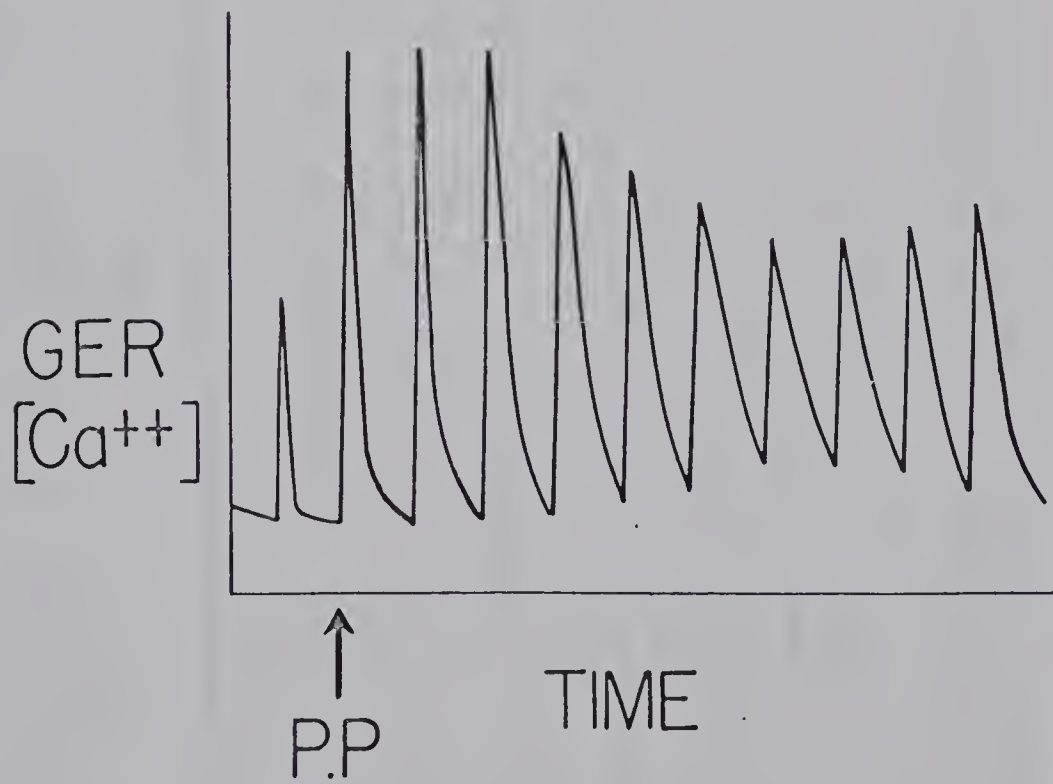
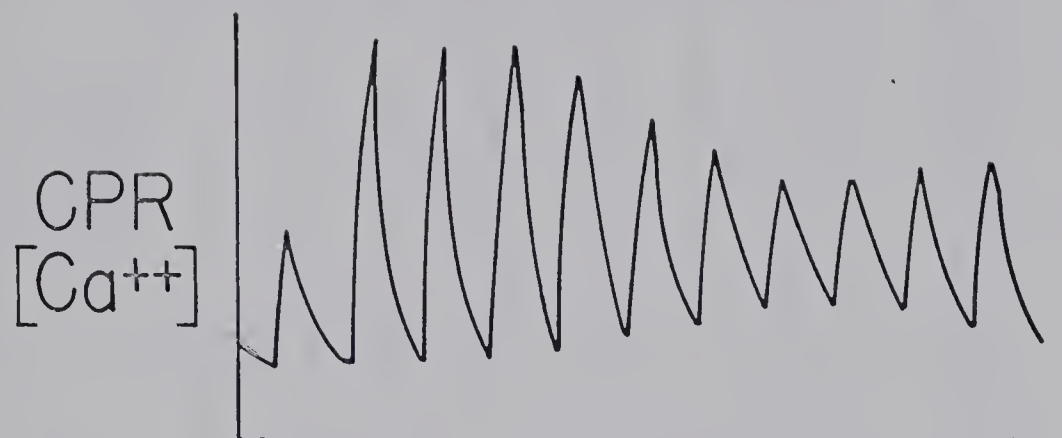


TABLE IV

Relationships Between Changes in Inotropism and Phosphorylase Activation

Equilibrating Calcium Ion Concentration (mM)	Stimulatory Parameter	Peak Systolic Tension (g)	Time to Peak Systolic Tension (sec)	Partial Contracture Evident	Phosphorylase Activation ^a (% Phosphorylase a)
0.6	P.P. ^b	14.3 ± 0.7	7.15 ± 0.28	—	11.9 ± 2.5 (@25"?)
1.2	P.P.	15.2 ± 0.9	3.80 ± 0.27	—	20.4 ± 3.4 (@15")
1.8	P.P.	16.2 ± 1.2	2.00 ± 0.20	+	32.7 ± 2.1 (@15")
0.6	EPI ^c	11.8 ± 0.8	23.2 ± 0.31	—	23.0 ± 3.1 (@20")
0.9	EPI	14.6 ± 0.6	19.4 ± 0.59	—	34.1 ± 1.7 (@20")
1.2	EPI	15.0 ± 0.5	18.0 ± 0.64	—	50.3 ± 4.5 (@20")
2.4	EPI	14.0 ± 0.8	15.2 ± 0.67	+	61.0 ± 4.0 (@25")

^a Maximal phosphorylase a values (± standard error of mean) are denoted, with the approximate time (in seconds) required for onset indicated in parenthesis.

^b Paired pulse stimuli, 300/min, 60 msec delay.

^c Epinephrine, 0.25 µg over a 5-sec interval.

CHAPTER X

The Significance of Calcium Ion in the Glycogenolytic Pathway of Striated Muscle: Conclusions

In Chapter III it was demonstrated that in the isolated perfused rat heart, it is not possible to produce a graded response, in terms of maximal systolic tension developed, by varying the delay period between paired stimuli. Brutsaert⁽⁵³⁾ did demonstrate a graded response to paired stimulation in the isolated cat papillary muscle. The studies described in Chapter III showed that the positive inotropic response seemed to be an all-or-none phenomenon (except for the vulnerable period), with maximal systolic tension always being developed whenever paired stimuli were effective in inducing positive inotropism. Positive inotropism under these conditions, was shown to precede an activation of phosphorylase.

In Chapter IV a closer time-course study of the positive inotropic events in relation to phosphorylase activation established that a significant portion of the calcium mobilized during the later, declining phase of the inotropic response to paired pulses, might be necessary for the phosphorylase activation observed.

Experiments described in Chapters V and VI showed that abruptly lowering the perfusing calcium ion concentration, so as to inhibit positive inotropism (absolute systolic tension) as normally induced by epinephrine or paired-pulse stimulation, is also effective in significantly inhibiting the phosphorylase activation normally observed. It was suggested that inhibition observed may be due to a prevention of the increased calcium mobilization (inwardly) normally associated

with maximal systolic tension development.

In Chapters VII and VIII it was shown that the degree of phosphorylase activation induced by paired stimulation or epinephrine administration decreases as the perfusing, equilibrated calcium ion concentration is decreased. It was surprising, however, to note that the maximal systolic tension developed is generally not reduced by this procedure; as it is when the perfusing calcium ion concentration is lowered abruptly (Chapters V and VI). Therefore, the argument explaining the inhibition of phosphorylase activation as observed in Chapters V and VI could not be applied to the situation in Chapters VII and VIII. A closer examination of the positive inotropic responses obtained to paired stimulation and epinephrine administration following equilibration with decreased calcium ion concentrations, it was revealed that the time to peak systolic tension was longer as the concentration employed was decreased. This thought-provoking observation led the author to formulate the model and hypothesis described in Chapter IX.

Controversy has arisen over whether calcium ion stimulates both the activity of inactivated and activated phosphorylase b kinase, or just the activity of activated phosphorylase b kinase. Drummond et al.⁽⁵¹⁾ have suggested that the former arrangement may in fact be applied to the in vivo situation in striated muscle. Recent work by Bromstrom and Krebs⁽¹⁶⁾, using purified preparations of phosphorylase b kinase, show that calcium ion does stimulate the activity of both forms of

phosphorylase b kinase at pH 8.2, although the inactivated kinase has a higher K_m value for calcium ($3 \times 10^{-6}M$) than does the activated kinase ($2 \times 10^{-7}M$). However, it is of interest to note that at the more physiological pH of 6.8, a K_m value for the inactivated phosphorylase b kinase was "indeterminate", while activated kinase has an only slightly increased K_m value ($5 \times 10^{-7}M$). Bromstrom and Krebs also demonstrated that "relaxing particles" had a calcium-reversible inhibitory effect on the activity of phosphorylase b kinase. The author is currently in support of the view that calcium ion has no physiological role for stimulating the activity of inactivated phosphorylase b kinase in vivo, and has therefore designed a number of experiments for testing such a hypothesis. Such experiments will entail the measurement of cyclic-AMP levels during the usage of parameters which affect calcium mobilization (see Addendum) and investigation of a possible potentiation of the phosphorylase-activating effect of epinephrine by electrical stimulation of striated muscle. Similar techniques to those described shall be employed, but the applicability of tissue slice techniques, along with possible electrophysiological measurements is currently being evaluated.

It should be appreciated that the in vivo picture of striated muscle glycogenolysis is probably much more complicated than it is presented in this thesis. Some investigators working in this field are studying the role of the phosphatases (the inactivating enzymes)

and their relation to muscle metabolism⁽⁶⁴⁾. The bio-energetics of the processes involved, with particular attention being paid to various nucleotides, is another area currently under investigation, and promises to be very revealing⁽⁶⁵⁾. As the sphere of knowledge expands it becomes increasingly complicated in its diversity. The author has presented his evidence in support of a role for calcium ion in cardiac glycogenolysis, but he does not hesitate to point out that calcium may be but a fraction of the whole.

Addendum.

It has been recently suggested ⁽⁶⁶⁾ that calcium ions may somehow affect the adenylyl cyclase system which modulates the glycogenolytic enzyme pathway. This has obvious implications with respect to the validity of the studies undertaken in this thesis. Therefore, it will be of interest to ascertain whether and to what degree, such an effect may be affecting the results observed in the foregoing Chapters.

Work has been begun on developing a method for assaying cardiac levels of cyclic-AMP. The method is essentially that as described by Brooker et al. ⁽⁶⁷⁾. At the time of this writing, however, successful employment of this technique has been postponed, and is pending the development of a suitable procedure for purifying cyclic-AMP from the tissue. Alternatively, newer techniques for assaying cyclic AMP are also currently under investigation.

BIBLIOGRAPHY

1. Cruickshank, E.W.H.; and Kosterlitz, H.W. The Utilization of Fat by the Aglycaemic Mammalian Heart. J. Physiol., 99:208 (1941).
2. Evans, C. Lovatt. The Effect of Glucose on the Gaseous Metabolism of the Isolated Mammalian Heart. J. Physiol., 47:407 (1914).
3. Wollenberger, A. Endogenous Catecholamine Mobilization and the Shift to Anaerobic Metabolism in the Acutely Ischaemic Myocardium, in the Symposium on the Coronary Circulation and Energetics of the Myocardium; Milan (1966), Basel, New York, 1967, S. Karger, p. 200.
4. Morgan, H.E.; and Parmeggiani, A. Regulation of Glycogenolysis in Muscle, in Whelan, W.J., and Cameron, M.P.; editors: "Control of Glycogen Metabolism" London, 1964, J. and A. Churchill, Ltd.; p. 254.
5. Morgan, H.E.; and Parmeggiani, A. Regulation of Glycogenolysis in Muscle. J. Biol. Chem., 239:2440 (1964).
6. Cornblath, M.; Randle, P.J.; Parmeggiani, A.; and Morgan, H.E. Regulation of Glycogenolysis in Muscle. J. Biol. Chem., 238:1592 (1963).
7. Williams, Betty J.; and Mayor, Steven E. Hormonal Effects on Glycogen Metabolism in the Rat Heart in Situ. Molec. Pharmacol., 2:454 (1966).
8. Hornbrook, K. Roger.; Quinn, Paul V.; Siegel, John H.; and Brody, Theodore M. Thyroid Hormone Regulation of Cardiac Glycogen Metabolism. Biochem. Pharmacol., 14:925 (1965).
9. Robison, G.A.; Butcher, R.W.; Dye, I.; Morgan, H.E.; and Sutherland, E.W. The Effect of Epinephrine on Adenosine 3',5'-Phosphate Levels in the Isolated Perfused Rat Heart. Molec. Pharmacol., 1:168 (1965).

10. Walsh, D.A.; Perkins, John P.; and Krebs, Edwin G. An Adenosine 3',5'-Monophosphate-dependant Protein Kinase from Rabbit Skeletal Muscle. J. Biol. Chem., 243:3763 (1968).
11. Fischer, Edmond H.; and Krebs, Edwin G. Conversion of Phosphorylase b to Phosphorylase a in Muscle Extracts. J. Biol. Chem., 216:121 (1955).
12. Newsholme, E.A. Regulation of Enzyme Activity. Sci. Progr., 53:237 (1965).
13. Cori, Gerty T.; and Cori, Carl F. The Kinetic of the Enzymatic Synthesis of Glycogen from Glucose-1-Phosphate. J. Biol. Chem., 135:733 (1940).
14. Mayer, S.E.; Williams, B.J.; and Smith, J.M. Adrenergic Mechanisms in Cardiac Glycogen Metabolism. Ann. N.Y. Acad. Sci., 139:686 (1967).
15. Morgan, H.E.; and Parmeggiani, A. Regulation of Glycogenolysis in Muscle. J. Biol. Chem., 239:2435 (1964).
16. Brostrom, Charles O.; and Krebs, Edwin G. The Regulation of Skeletal Muscle Phosphorylase b Kinase by Ca^{++} . Fed. Proced., 1970, 2648 (Abs).
17. The Handbook of Biological Data, Williams S. Spector, editor. Philadelphia, 1961, W.B. Saunders Co. p. 68.
18. Drummond, George I.; and Duncan, Loverne. Activation of Cardiac Phosphorylase b Kinase. J. Biol. Chem., 241:5893 (1966).
19. Langer, G.A. Ion Fluxes in Cardiac Excitation and Contraction and Their Relation to Myocardial Contractility. Physiol. Rev., 48:708 (1968).
20. Katz, Arnold M. Contractile Proteins of the Heart. Physiol. Rev., 50:63 (1970).
21. Winegrad, Saul; and Shanes, Abraham M. Calcium Flux and Contractility in Guinea Pig Atria. J. Gen. Physiol., 45:371 (1961).

22. McLean; and Hastings. Clinical Estimation and Significance of Calcium Ion Concentrations in the Blood. Am. J. Med. Sci., 189:601 (1935).
23. Neuman, W.F.; and Neuman, M.N. The Chemical Dynamics of Bone Mineral, 1958, University of Chicago Press.
24. Marsh, B.B. The Effects of Adenosine Triphosphate on the Fibre Volume of a Muscle Homogenate. Biochim. Biophys. Acta, 9:247 (1952).
25. Portzehl, H. Die Binding des Erschlaffungs faktors an die Muskelgrana. Biochim. Biophys. Acta, 26:373 (1957).
26. Hasselbach, W.; and Makinose, M. Die Calciumpumpe der "Erschlaffungsgrana" des Muskels und ihre Abhängigkeit von der ATP-Spaltung. Biochem. Z., 333:518 (1961).
27. Ebashi, Setsuro; and Lipmann, Fritz. Adenosine Triphosphate-Linked Concentration of Calcium Ions in a Particulate Fraction of Rabbit Muscle. J. Cell Biol., 14:389 (1962).
28. Weber, Annemarie; Herz, Ruth; and Reiss, Ingrid. On the Mechanism of the Relaxing Effect of Fragmented Sarcoplasmic Reticulum. J. Gen. Physiol., 46:679 (1963).
29. Fanburg, B.; Finkel, R.M.; and Martonosi, A. The Role of Calcium in the Mechanism of Relaxation of Cardiac Muscle. J. Biol. Chem., 239:2298 (1964).
30. Katz, Arnold M.; Repke, Doris I. Quantitative Aspects of Dog Cardiac Microsomal Calcium Binding and Calcium Uptake. Cir. Res., 21:153 (1967).
31. Palmer, Roger F.; and Posey, Virginia A. Ion Effects on Calcium Accumulation by Cardiac Sarcoplasmic Reticulum. J. Gen. Physiol., 50:2085 (1967).
32. Weber, Annemarie; Herz, Ruth; and Reiss, Ingrid. The Nature of the Cardiac Relaxing Factor. Biochim. Biophys. Acta, 131:188 (1967).

33. Krebs, Edwin G.; Graves, Donald J.; and Fischer, Edmond H. Factors Affecting the Activity of Muscle Phosphorylase b Kinase. J. Biol. Chem., 234:2867 (1959).
34. Meyer, William L.; Fischer, Edmonton H.; and Krebs, Edwin G. Activation of Skeletal Muscle Phosphorylase b Kinase by Ca^{2+} . Biochem. (A.C.S.), 3:1033 (1964).
35. Hammermeister, K.E.; Yunis, A.A., and Krebs, E.G. Studies on Phosphorylase Activation in the Heart. J. Biol. Chem., 340:986 (1965).
36. Drummond, George I.; and Duncan, Loverne. On the Mechanism of Activation of Phosphorylase b Kinase by Calcium. J. Biol. Chem., 243:5532 (1968).
37. Huston, R.B.; and Krebs, E.G. Activation of Skeletal Muscle Phosphorylase Kinase by Ca^{2+} . II. Identification of the Kinase Activating Factor as a Proteolytic Enzyme. Biochem. (A.C.S.), 7:2116 (1968).
38. Hangaard, Niels. Role of the Phosphorylase Enzymes in Cardiac Contraction: A Proposed Theory for the Rhythmical Production of Energy in the Heart. Nature (L), 197:1072 (1963).
39. Belford, Julius; and Feinleib, Marcia R. Phosphorylase Activity of Heart Muscle Under Various Conditions Affecting Force of Contraction. J. Pharmacol. Exp. Therap., 127:257 (1959).
40. Mayer, Steven E.; and Moran, Neil C. Relation Between Pharmacological Augmentation of Cardiac Contractile Force and the Activation of Myocardial Glycogen Phosphorylase. J. Pharmacol. Exp. Therap., 129:271 (1960).
41. Hess, Marilyn E.; Shanfeld, Joseph; and Haugaard, Niels. The Role of the Autonomic Nervous System in the Regulation of Heart Phosphorylase in the Open-Chest Rat. J. Pharmacol. Exp. Therap., 135:191 (1962).

42. Mayer, Steven E.; Cotten, Marion deV; and Moran, Neil C.
Dissociation of the Augmentation of Cardiac Contractile Force
from the Activation of Myocardial Phosphorylase by Catecholamines.
J. Pharmacol. Exp. Therap., 139:275 (1963).
43. Drummond, George I.; Valadares, J.R.E.; and Duncan, Loverne.
Effect of Epinephrine on Contractile Tension and Phosphorylase
Activation in Rat and Dog Hearts. Proc. Soc. Exp. Biol. Med.,
117:307 (1964).
44. Williamson, J.R.; and Jamieson, Mrs. D. Dissociation of the
Inotropic from the Glycogenolytic Effect of Epinephrine in the
Isolated Rat Heart. Nature (L), 206:364 (1965).
45. Robison, G.A.; Butcher, R.W.; Oye, I.; Morgan, H.E.; and
Sutherland, E.W. The Effect of Epinephrine on Adenosine 3',5'-
Phosphate Levels in the Isolated Perfused Rat Heart. Molec.
Pharmacol., 1:168 (1965).
46. Valadares, J.R.E.; and Friesen, Abram J.D. Time Course of
Epinephrine-Induced Activation of Phosphorylase in Rat and Dog
Hearts. Life Sci., 5:521 (1966).
47. Friesen, Abram J.D.; Allen, Glenna; and Valadares, Joseph R.E.
Calcium-Induced Activation of Phosphorylase in Rat Hearts.
Sci., 155:1108 (1967).
48. Bhagat, B.; Saini, N.; West, W.L.; and Booker, W.M. Modification
of Action of Sympathomimetic Amines by Triiodothyronine. Arch.
Int. Pharmacodyn., 155:126 (1965).
49. Namm, Donald H.; Mayer, Steven E.; and Maltbie, Margaret. The
Role of Potassium and Calcium Ions in the Effect of Epinephrine
on Cardiac Cyclic Adenosine 3', 5'-Monophosphate, Phosphorylase
Kinase, and Phosphorylase. Molec. Pharmacol., 4:522 (1968).
50. Friesen, Abram J.D.; Oliver, Neil; and Allen, Glenna. Activation
of Cardiac Glycogen Phosphorylase by Calcium. Am. J. Physiol.,
217:445 (1969).

51. Drummond, George I.; Harwood, James P.; and Powell, C. Anne. Studies on the Activation of Phosphorylase in Skeletal Muscle by Contraction and by Epinephrine. J. Biol. Chem., 244:4235 (1969).
52. Posner, Jerome B.; Stern, Robert; and Krebs, Edwin G. Effects of Electrical Stimulation and Epinephrine on Muscle Phosphorylase, Phosphorylase b Kinase, and Adenosine 3',5'-Phosphate. J. Biol. Chem., 240:982 (1965)
53. Brutsaert, D.L. Studies on the Potentiation of Contractility of Heart Papillary Muscle by Paired Stimulation. Arch. Int. Physiol. Biochim., 74:642 (1966).
54. Cori, Gerty.; and Illingworth, Barbara. The Effect of Epinephrine and Other Glycogenolytic Agents on the Phosphorylase a Content of Muscle. Biochim. Biophys. Acta, 21:105 (1956).
55. Mayer, S.W.; Cotten, M.D.; and Moran, N.C. Dissociation of the Augmentation of Cardiac Contractile Force from the Activation of Myocardial Phosphorylase by Catecholamines. J. Pharmacol. Expt. Therap., 139:275 (1963).
56. Suko, Josef; Ueba, Yasuyuki; and Ainsley, Charles A. Intracellular Calcium and Myocardial Contractility, II. Effects of Postextrasystolic Potentiation in the Isolated Rabbit Heart. Cir. Res., 27:227 (1970).
57. Wood, Earl H.; Heppner, Richard L.; and Weidmann, Silvio. Inotropic Effects of Electric Currents, I. Positive and Negative Effects of Electric Currents or Current Pulses Applied During Cardiac Action Potentials, II. Hypothesis: Calcium Movements, Excitation-Contraction Coupling and Inotropic Effects. Cir. Res., 24:409 (1969).
58. Mines, G.F. On Circulating Excitation in Heart Muscle, and Their Possible Relation to Tachycardia and Fibrillation. Trans. Roy. Soc. Can., 8:43 (1914).

59. Wegeria, R.; Moe, G.K.; and Wigger, C.J. Comparison of the Vulnerable Periods and Fibrillation Thresholds of Normal and Idioventricular Beats. Am. J. Physiol., 133:651 (1941).
60. Spiro, David and Sonnenblick, Edmund H. Comparison of the Ultrastructural Basis of the Contractile Process in Heart and Skeletal Muscle. Supplement II to Cir. Res., 14 and 15:14 (1964).
61. Forssmann, W.G. and Girardier, L. A Study of the T-System in Rat Heart. J. Cell. Biol., 44:1 (1970).
62. Porter, Keith R. and Palade, George E. Studies on the Endoplasmic Reticulum, III. Its Form and Distribution in Striated Muscle Cells. J. Biophys. Biochem. Cytol., 3:269 (1947).
63. Meyer, Francois; Heilmeyer Jr., Ludwig M.G.; Haschke, Richard H.; and Fischer, Edmond H. Control of Phosphorylase Activity in a Muscle Glycogen Particle, I. Isolation and Characterization of the Protein-Glycogen Complex. J. Biol. Chem., 245:6642 (1970).
64. Haschke, Richard H.; Heilmeyer Jr., Ludwig M.G.; Meyer, Francois; and Fischer, Edmond H. Control of Phosphorylase Activity in a Muscle Glycogen Particle, III. Regulation of Phosphorylase Phosphatase. J. Biol. Chem., 245:6657 (1970).
65. Villar-Palasi, C. and Wei, S.H. Conversion of Glycogen Phosphorylase b to a by Non-Activated Phosphorylase b Kinase: an In Vitro Model of the Mechanism of Increase in Phosphorylase a Activity with Muscle Contraction. Proc. Nat. Acad. Sci, 67: 345 (1970).
66. Brooker, Gary; Thomas, Jr., Lyell J.; and Appleman, M. Michael. The Assay of Adenosine 3', 5'-Cyclic Monophosphate and Guanosine 3', 5'-Cyclic Monophosphate in Biological Materials by Enzymatic Radioisotope Displacement. Biochem., 7:4177 (1968).
67. Rasmussen, Howard. Cell Communication, Calcium Ion, and Cyclic Adenosine Monophosphate. Sci., 170:404 (1970).

"Sweet is the lore which Nature brings;
Our meddling intellect
Misshapes the beauteous forms of things:--
We murder to dissect.

Enough of science and of art;
Close up these barren leaves;
Come forth, and bring with you a heart
That watches and receives."

William Wordsworth.

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